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From the Department of Physiology Karolinska Institutet, Stockholm, Sweden

The First Forty Years of the Scandinavian Physiological Society

A review given at Turku University in connection with the opening of the 12th Scandinavian Congress of Physiology in Turku, August 22 1966

By

C. G. Bernhard

One of the tasks of the Scandinavian Physiological Society according to its constitution, is to arrange a congress in physiology every third year enabling the members from Scandinavian countries t exchange the results of their research, and to discuss common concerns and personal matters. Our hosts in Turku have diligently noticed that the Society is now 40 years old and because of this they have given me the pleasure of reviewing its history

One should first mention that it is now exactly 50 years since the thought of a Scandinavian physiological society first cam up. It was actually at the Scandinavian Nature Research Meeting (Skandinaviska N turforskarmötet) in Kristiania in 1916 that the idea was first put forth. The suggestion was made by the Norwegian physiologist Sophus Torup whose name is associated with being a scientific consultant for Frithiof Nansen's polar expedition. Robert Tigerstedt from Helsinki, whose word carried much weight, backed the suggestion, but the current political situation was such that making a reality out of the idea was impossible. It was the Lund scientists who caught the ball when their physiological society because of its 10th anniversary in April 1925 initiated the first Scandinavian Congress in Physiology and Experimental Medicine. The president of the congress, Torsten Thunberg, expressed in his opening remarks the view that both the high attendance and th quality of the scientific contributions could be taken as evidence for the consolidation of the physiologists' position in Scandinavia. There was, he meant, no longer any reason to have the pessimistic approach that Berzelius had expressed in a letter to Örstedt written in 1839 when considering the possibility of holding a conference in natural science

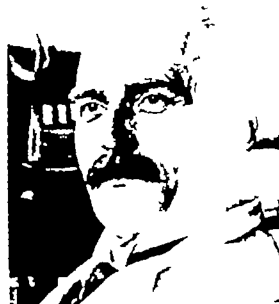
W are entirely too few in number to play at scientific meetings, to imitate the Germans and Englishmen. If one could hunt up 30—50 scientists in different fields by diligently writing letters I think one would be doing well. In Sweden scientists are so poorly paid that they consider themselves lucky if their debits and credits cancel each other at the end of each year using their strictest economy And they don t have money to tra el to meetings. If they came once, they would certainly never be ble to come again

In connection with the congress in Lund, under Torsten Thunberg s chairmanship, n inaugural session was held with Gunnar Ahlgren at the minutes. The society's



G Liljestrand

Fig. 1. *Göran Liljestrand* (born 1886) professor of pharmacology at Karolinska Institutet (1927—1951) one of the founders of the Scandinavian Physiological Society and honorary member of the Society since 1951. Liljestrand was the secretary of the Society 1926—1948, succeeded by C. G. Bernhard, Stockholm (1948—1963) and Bengt Andersson, Stockholm since 1963. He was also editor of Skandinavisches Archiv für Physiologie (1939—1940) and of Acta Physiologica Scandinavica (1940—1957) succeeded by Ulf von Euler since 1957.



Sophus Torup

Fig. 2. *Sophus Torup* (1861—1937) professor of physiology at Oslo University (1889—1931)



Fig 3 *Robert Tigerstedt* (1853—1923) professor of physiology at Karolinska Institutet (1886—1900) and at Helsinki University (1900—1919) Tigerstedt was editor of Skandinavisches Archiv für Physiologie (1897—1923) after Frithiof Holmgren (1889—1897) and succeeded by C. G. Santesson (1923—1939) The portrait is painted by his daughter in law Heloy Tigerstedt at the centennial in 1953



Fig 4 *Torsten Thunberg* (1873–195. professor of physiology Lund University 1905–1938

name which should signify the lines of its future endeavour was suggested by August Krogh after a lively debate on how the interests of the adjacent scientific fields should be best protected. The problem was solved in the constitution. Professor Jöns Johansson, in Stockholm, together with docent Göran Liljestrand, was given the assignment of drafting the constitution. Their proposals were passed without change at the Society's first meeting which was held one year later in connection with the international physiology congress in Stockholm in August 1926 at which occasion Liljestrand was elected secretary general.

It is not only because of the coincidence that Göran Liljestrand, professor emeritus and honorary member of the Society just now happens to be twice as old as the Society that I am taking the opportunity of especially haranguing him, but more importantly I want to express our gratitude to him for his contributions to Scandinavian physiology and our happiness at seeing him here in this circle where his vivacious personality is an enduring source of inspiration.

A rule was passed that congress should be held every third year. The meetings should rotate through the Scandinavian countries, each one of which should be represented on the central board. Transactions from the meetings describe how the Society has fullfilled its tasks through the congresses. They also show changes with the generations: they mirror the actualities of the time and give a review of the development of physiological research in the Scandinavian countries during the past half-century. The high standard of the scientific contributions at the Lund meeting in 1925 set a precedence for future work. Thunberg's lecture on the dehydrogenase system, Overton's demonstration of the mechanisms for the action of narcotics on nerves and Widmark's presentation of his method for estimating the alcohol content in blood, all represent fundamental contributions of undiminished relevance from three renowned scholars in Lund. Thunberg also demonstrated his respirator. Today when the technical help of film, TV and tape is also taken for granted in academic education, I like to mention August Krogh's lecture on cinematographic technique in research and teaching. This was indeed the technique he used in his classical investigations on capillary circulation for which he was awarded the Nobel prize in 1920.

In our times of debates on the role of the sexes it is perhaps worthwhile to recall Sven Ingvar's lecture for the ladies of the congress, "On Woman's Nervous System". Widmark's informal talk, "On the Colours of Our Food" formed a transition to the no doubt high class culinary part of the program where the menu's finale "bombe vitaminique" gave a certain compensation for the poorly presented field of vitamin research in the scientific program.

However, this point was soon altered. During the following congresses, up to 1937, the number of lectures in biochemistry increased, especially those in the fields of enzyme and vitamin research. When the second congress was held in Oslo in 1928 Hans von Euler discussed the enzymatic breakdown of sugar investigations which led to this Nobel prize the following year. In the congressional records for 1928 the next paragraph concerns an investigation of adrenaline by his son Ulf von



Fig. 5. *August Krogh* (1874—1949) professor of zoophysiology at Copenhagen University (1916—1945). After that Krogh continued his scientific work in a private laboratory at Gjentoft supported by the Carlsberg and Scandinavian Insulin Foundations. In 1920 Krogh was awarded the Nobel prize in physiology or medicine "for his discovery of the capillary motor regulating mechanism".



Fig. 6. *Jöns Johansson* (1862—1938) professor of physiology at Karolinska Institutet (1901—1927).

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From the Department of Physiology Karolinska Institutet, Stockholm, Sweden

Afferent Activity Recorded in the Kidney Nerves of Rats

By

A. Åström and J Crafoord

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Abstract

Åström A. and J Crafoord. *Afferent activity recorded in the kidney nerves of rats* Acta physiol scand. 1967 *70* 10—15

In anesthetized rats no spontaneous afferent activity in the kidney nerves could be recorded. If the renal vein pressure was elevated, or if i.v. infusion of saline or Ringer solution was given an afferent discharge of a slowly adapting type could be recorded. *It is concluded that an adequate* stimulus for the mechanoreceptors responsible for this activity is increased kidney tenseness (intrarenal pressure) and perhaps also distension of the intrarenal veins. The results indicate that more than one kind of fibers are engaged in the afferent activity which can be elicited by various kinds of stimuli.

As part of a study of the renal autoregulation of blood flow some factors determining kidney tenseness (intrarenal pressure) have previously been investigated in cat (Åström and Samelius 1962). From these studies it was concluded that the intrarenal pressure could constitute a factor in the autoregulation of blood flow in the normally innervated kidney. However it was questioned whether such a factor — and differences in the prevailing vasomotor tone — could fully explain the variations in pressure-flow relationships observed in different kidneys.

One possibility would seem to be that kidney tenseness could influence the vasomotor tone in the kidney by way of a reflex mechanism. It is well known that the kidneys are abundantly supplied with autonomic nerves but the possibility that some of these might be afferent seems to have attracted little attention. The only studies concerning afferent impulses in the kidney nerves that we are aware of are those by Pines (*e.g.* 1960). In cats he found that afferent activity could be recorded which increased in intensity *e.g.* when the venous pressure was elevated.

In the present investigation afferent impulses have been recorded in the kidney nerves of rats. The rat was chosen as experimental animal mainly since it was being used in our laboratory for studies of the possible influence of efferent nerves on sodium excretion and other kidney function.

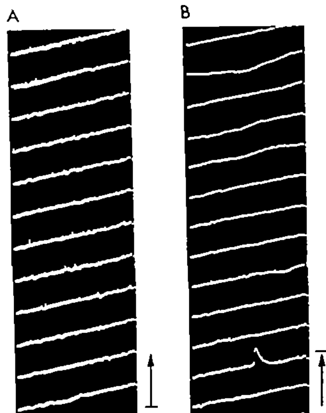


Fig 1 Afferen activity from the kidney nerv in rats. Recordings should be read from below upw rds. Each sweep corresponds to 200 msec.
A. Increasing the enous pressure to bout 70 mm Hg by partial occlusion of the renal ein elicits afferent impulse discharge
B After release the activity disappears within less than 1 sec

Methods

The experiments were carried out on rats (Sprague Dawley 200—250 g) anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally The trachea was cannulated and the left kidney exposed by mid-line incision Systemic blood pressure was measured by catheter in the right femoral artery renal ein pressure from catheter in the spermatic (ovarian) vein and injections were made in the righ jugular ein.

The kidney nerves were prepared using binocula dissecting microscope and isolated from the hilus in the proximal direction to the level of the arterial branch he suprarenal gland and cu Silver electrodes, as rule monopolar ere used for the recording of afferent nerv activity from the peripheral stump of the nerv A Grass P 6 preamplifier Tektronix 502 A oscilloscope and Grass model C-4 camera were used for the recording of the action potentials. Mainly to check the general condition of the animal Grass model 5 polygraph was used in parallel with the cathode ray oscilloscope t record systemic arterial nd renal ein pressure heart ra and urine flow (photoelectric drop counter) I the latter case he ureter was cannulated with polyethylene tube (PE 50) introduced in the renal pelvis

Venous pressure was elevated by gentle pressure on the renal ein pplied with glass rod. Intravenous infusions were done with motor-driven syringe t speed of usually 0 2 ml per min.

Results

In the series of about 40 rats included in this study spontaneous afferent activity in the kidney nerves could never be definitely demonstrated. If, however the pressure in the renal ein was elevated by stasis produced by gentle pressure on the vein with a glass rod (VP = 10 — 30 mm Hg) afferent activity could be recorded in about

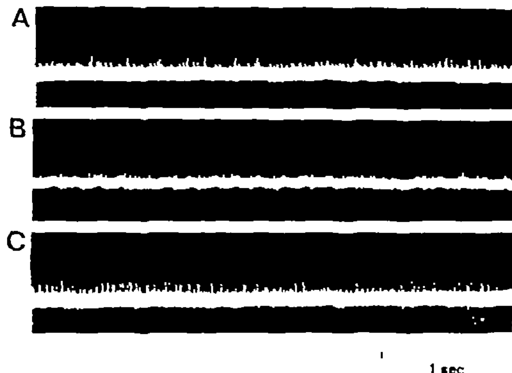


Fig 2. Rat 250 g Afferent impulses in the kidney nerve.
A. Effect of renal vein stasis at the beginning of the experiment.
At normal venous pressure no spontaneous activity (not shown in the figure)
B. After the i. infusion of 10 ml of saline over period of 45 min some spontaneous activity is t normal venous pressure
C. Elevation of venous pressure at this point produces intense activity

half of the cases. The response illustrated in Fig 1 was obtained within 1—2 sec after the application of the stasis. The response showed some degree of adaptation during the test period of ten sec and the spikes were of relatively large amplitude. In several other experiments another type of response was observed which tended to occur after a somewhat longer delay and to subside more gradually. This type of response showed no certain adaptation during 10—20 sec and the action potentials were of relatively low amplitude (Fig 2 A). It has not been possible to determine the conduction velocity of the fibres concerned.

Intravenous infusion of saline has previously been found to increase the subcapsular pressure in the kidney. In the present study infusion of saline or Ringer solution at a rate of about 5 ml over a period of 20 min sometimes elicited afferent activity of low frequency (Fig 2, B). After these infusions venous stasis often produced a much more pronounced effect (Fig 2 C) than before the infusion (Fig 2 A).

Intraarterial injection of small volumes of saline or Ringer solution causes a transient increase in subcapsular pressure (previous observations). Injection of a few ml of saline or Ringer solution intraarterially via a catheter introduced from the femoral artery to the level above the renal artery was also found to produce an

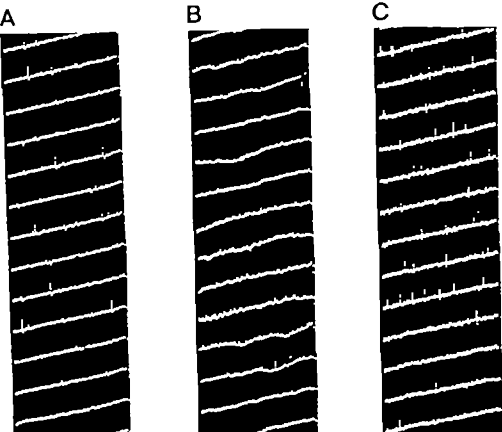


Fig 3 Afferent impulses in the kidney nerve. Each sweep 200 msec 2 ml of Ringer solution injected locally in the renal artery elicits afferent impulses (A) while the same injection i.v. is ineffective (B)
C The effect of mechanical pressure applied to the hilus area of the kidney parenchyma.

afferent discharge (Fig 3 A) The same injection (2 ml Ringer solution) given i.v. had no such effect (B) Increasing the subcapsular pressure by infusion of mannitol (5 ml of 25 % solution in 20 min) which had a marked diuretic effect, also tended t augment the response to elevation of venous pressure.

In an attempt to localize the receptors for the afferent activity pressure with a glass rod was applied to the ureter instead of the renal vein. In some instances this procedure elicited afferent activity Pinching the ureter with a pair of forceps gave similar results. The afferent activity elicited from the ureter was different from that obtained by venous stasis and became apparent mainly as a widening of the baseline. Distension of th renal pelvis with saline (50 mm Hg) did not elicit any definite response

Pressure pplied with the glass rod on the kidney surface was ineffective except when t was applied to rather small area of the kidney parenchyma at the hilus of the kidney In the experiments in which this test was positive (Fig 3 C) the action

potentials evoked seemed to be of the same type as those elicited by elevation of renal vein pressure in the same preparation.

If strong pressure was applied to the vein and particularly to the ureter reflexly evoked muscle activity would often interfere with the recording. This influence originating from the caudal musculature was unexpectedly strong, probably due to the fact that these muscles in the rat are well developed for the control of the tail. The muscle interference was easily elicited by arousing the animal with painful stimuli like pinching a foot. To distinguish the muscle interference from the nerve activity the electromyogram was often recorded on the other channel of the oscilloscope. Curarizing the animal abolished the muscle interference but not the afferent nerve activity produced by venous stasis or mechanical pressure applied to the hilus area of the kidney surface.

Discussion

The experiments have shown that afferent impulses can be recorded in the kidney nerves during conditions of elevated renal vein pressure but not under the ordinary experimental conditions in the anesthetized rat. In some instances (cf. Fig. 1) the response elicited was rapid in onset and consisted of large spikes of short duration. In the majority of cases, however, the action potentials appeared to be of somewhat longer duration and of lower amplitude indicating fibres of smaller size. The i.v. infusion of large volumes of saline or Ringer solution often evoked responses similar to those observed after venous stasis.

It would not seem unlikely that more than one type of mechanoreceptors are present in the kidney. The response with large spikes of short latency following the application of venous stasis may well originate from the walls of the intrarenal veins or their close proximity. Distension of intrarenal veins would seem to be the adequate stimulus for these receptors. Application of gentle pressure to the hilus area of the kidney parenchyma, which as a rule elicited an afferent discharge in cases in which venous pressure elevation was effective, would also seem to activate this type of receptors.

The other type of afferent response seemed to build up more gradually following the application of venous stasis and did not show any definite adaptation during a 10—20 sec test period. This type of response was indistinguishable from that sometimes elicited by intravenous infusion of large volumes of saline or by small amounts of saline injected locally in the renal artery. It would seem that the common cause for this latter type of response was an increase in kidney tenseness (intrarenal pressure) and this may therefore be suggested as being the adequate stimulus for this type of mechanoreceptors in the kidney.

The afferent discharge sometimes recorded when the ureter was pinched or compressed with the glass rod seemed to be different from that discussed above. The action potentials were much smaller and broader and probably originated from so-called visceral pain afferents.

Pines (1960) observed that the afferent impulses in the kidney nerves in cats decreased with a fall in arterial pressure and increased when the aorta below the renal artery was clamped or when 100 ml saline was injected intravenously. He also found that electrical stimulation of vasoconstrictor nerves or high doses of adrenaline reduced the afferent activity. In the study by Åström and Samelius (1962) the tense-

ness of the kidney was found to increase with renal arterial pressure and to decrease during electrical stimulation of the efferent renal nerves and following the injection of adrenaline. Pines suggested that the afferent discharge originated from different kinds of receptors and was determined by the blood-filling of the kidneys which in turn varied with changes in the general circulation.

The finding that afferent information concerning the tenseness of the kidney is transmitted centrally would seem to be of interest for the discussion of the local adjustments of kidney circulation and the autoregulation of kidney blood flow in the normally innervated kidney. This kind of afferent information would also possibly be suitable for the control of the extravascular fluid volume in a more general way. Some further studies of the afferent impulses from the kidney are being made in cats.

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From the Nobel Institut for Neurophysiology Karolinska Institutet, Stockholm Sweden and the Department of Physiology University of Umeå, Sweden

The Effect of Calcium on Potassium Contractures of Single Slow Muscle Fibres of Xenopus Laevis

By

JAN LÄNNERGREN

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Abstract

LÄNNERGREN J. *The effect of calcium on potassium contractures of single slow muscle fibres of Xenopus laevis.* Acta physiol. scand. 1967. 70. 16—25.

Isolated slow muscle fibres of Xenopus laevis were subjected to step increases in external potassium concentration at various external calcium concentrations. An increase in $[Ca]_o$ *shifted the curve relating peak tension to log* $[K]_o$ *to higher K-concentrations, a decrease in* $[Ca]_o$ *caused a shift in the opposite direction. Similar effects were seen on the relation between inactivation and log* $[K]_o$. *The peak tension was but little affected by* $[Ca]_o$*; the time-course of contractures was altered in such a way that the rate of relaxation was increased in low* $[Ca]_o$ *and decreased in high* $[Ca]_o$. *Evidence was obtained that the effect of altered* $[Ca]_o$ *occurs as rapidly as that of altered* $[K]_o$. *Some observations were also made on contractures in Ca-free solutions and in solutions with Na partially substituted by Ba.*

There is evidence that calcium exerts an effect upon at least two systems involved in the control of muscular contraction. The one is a direct effect upon the contractile mechanism *per se* as evidenced by the calcium-dependence of the interaction of isolated actomyosin systems with ATP (Weber, Herz and Reiss 1963 *a, b*). The other effect is exerted on the membrane of the muscle cell and involves an alteration of the relation between tension production and membrane potential. Lüttgau 1963 and Frankenhaeuser and Lännergren 1967 have shown that the mechanical threshold and the rate of rise and fall of tension in a potassium contracture of a twitch muscle fibre are all influenced by the concentration of external calcium.

Slow muscle fibres have a surface membrane which does not show regenerative electrical activity and consequently does not conduct action potentials. The results of some investigation indicate that the effect of calcium on these fibres is different from that on twitch fibres. In experiments on potassium contractures in single slow fibres from the iliofibularis muscle of the frog Lüttgau (1963) showed that a maintained contracture could be elicited by increased [K] in solutions with low as well as with high [Ca]. Larger tensions were obtained in solutions with high [Ca]

Similar results were obtained by Schaechtelin (1961) on strips of frog rectus abdominis muscle and by Aidley (1965) on a locust slow muscle. The findings of Schaechtelin further indicate a shift of the tension — log $[K]_o$ curve in a direction opposite to that seen with twitch fibres. *The results of other investigations show similar effects of* calcium on slow and fast fibres. For example, Pauschinger and Brecht (1961) found that $[Ca]_o$ influenced potassium contractures of bundles of either slow or fast fibres (m. iliofibularis) in the same way: the relaxation of both slow and fast fibres was rapid in the high-K solution after treatment with a Ca-free solution. In contrast to the findings of Lüttgau (1963) these authors saw but a small effect of $[Ca]_o$ on the maximal tension of the slow fibres.

Because of these contradictory results it was of interest to reinvestigate the problem of the effect of calcium on slow muscle fibres. Single fibres were used in order to avoid long diffusion times.

In a previous paper (Lännergren 1967) it was shown that slow fibres from the iliofibularis muscle of *Xenopus laevis* gave long-lasting contractures in 15—80 mM K solutions and that the threshold for initiation of contracture was exceedingly well defined. The present paper describes the effect of changes in $[Ca]_o$ on the mechanical threshold and on the amplitude and time-course of the contractures and shows that these effects are comparable to those on twitch fibres. Some experiments were also made to determine the speed of the effect of altered $[Ca]_o$. It will be shown that the effects occur very rapidly. The main conclusion drawn is that the effects of calcium described here are exerted on the membrane and not on the contractile system proper.

Methods

Isolated slow muscle fibres from the iliofibularis muscle of *Xenopus laevis* were used. Rapid changes of the external solution were made while the tension in the fibre was recorded as described previously (Frankenhaeuser and Lännergren 1967). The experiments were performed between October and March at room temperatures between 20 and 24° C.

Solutions. The normal Ringer's solution had the composition (mM): NaCl 112.0, KCl 2.5, $NaHCO_3$ 2.5, $CaCl_2$ 2.0, Na_2HPO_4 1.2, NaH_2PO_4 0.6. It was continuously bubbled with a mixture of 99 % O_2 and 1 % CO_2; pH was 7.2—7.4. Ringer's solutions in which [Ca] was altered contained no phosphate buffer. Osmolarity was maintained by adjustment of NaCl concentration. Test solutions contained various amounts of KCH_3SO_4 (Hopkin & Williams, Ltd., England) in exchange for equimolar amounts of NaCl. No special precautions were taken in preparing the Ca-free solutions. Glass-distilled water was used throughout.

Results

The effect of altered $[Ca]_o$ on the mechanical threshold and on the time-course of the contracture

The effect of altered $[Ca]_o$ on contractures evoked by various K-concentrations was studied in nine fibres. The procedure was the following. Between tests the fibre was surrounded by slowly-flowing Ringer's solution. Two min before the test a change was made to a Ringer's solution with the [Ca] to be used during the following test and the flow of solution was made more rapid. In normal $[Ca]_o$ (2mM) contractures began at about 15 mM K and reached their maximum at 20 mM K. Further increase in K-concentration did not lead to an increase in peak tension (see also Lännergren

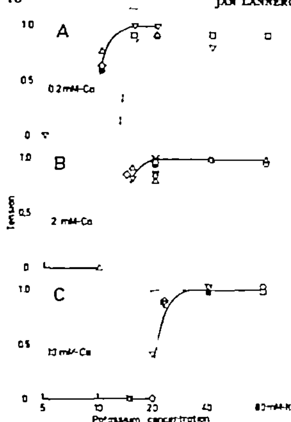


Fig 1 Relation between [K] (logarithmic scale) and peak tension at three different $[Ca]_o$. Ordinates are given as fractions of peak tension of first contracture (in 20 mM K, mM-Ca, ×). Other values in 20 mM K, mM-Ca are controls. A 0.2 mM-Ca in equilibrating and test solutions. B normal $[Ca]_o$ (2mM). C, 10 mM Ca. fibre 3, diameter 60 μm maximal tension 3. kg cm²; ● fibre 8, 40 μm, 3.0 kg/cm; △ fibre 1, 5 μm, 4.8 kg cm; ▽ fibre 13, 70 μm, 3.9 kg cm; □, fibre 16, 40 μm, 3.5 kg cm; ○ fibre 20, 90 μm, 3.9 kg cm

1967) instead the tension was less well maintained, falling to about 50 of the initial value in 30 sec in 80 mM K.

When 10 mM Ca was used in the equilibration and test solutions no tension developed in 15 mM K. In 20 mM K the fibres either were not activated or more commonly tension developed relatively rapidly after an initial delay of 10—20 sec A few fibres showed a slow increase in tension. Maximum tension was reached in solutions with 40 mM K or more. The effect of decreased $[Ca]_o$ (0.2 mM) was that contractures were elicited even at 10 mM K and maximum tension was reached in solutions with 15 mM K Fig 1 is a plot of the results from six fibres. A smooth curve was drawn through the points obtained in 2 mM-Ca and the same curve was used to fit the values obtained in 0.2 and 10 mM-Ca by shifting it along the horizontal axis. The curve was not drawn in full between zero tension and the lowest tension values since no contractures were ever obtained with tension in this intermediate range. Membrane potential was not measured and therefore the shift cannot be expressed as potential. It is nevertheless apparent from Fig 1 that the effect of a five fold increase in $[Ca]_o$ was larger than that of a ten-fold reduction.

In the higher K-concentrations the tension decreased with time In increased $[Ca]_o$ this decay was absent or slower at corresponding [K] (Fig 2 A) while a decrease in $[Ca]_o$ had the opposite effect Fig 2 B

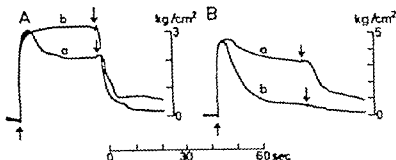


Fig. 2. Effect of $[Ca]_o$ on time-course of contracture. *A*, two contractures in 40 mM-K, *a*, in 2 mM-Ca, and *b* in 10 mM-Ca. No relaxation at this $[K]_o$ in high $[Ca]_o$. Photographical superposition of tension records. Fibre 3, diameter 60 µm, maximal tension 3.2 kg/cm². *B*, two photographically superimposed tension records of contractures in 40 mM-K, 2 mM-Ca (*a*) and in 40 mM-K, 0.2 mM-Ca (*b*). Increased rate of relaxation in low $[Ca]_o$. Fibre 28, diameter 50 µm, 5.0 kg/cm². Fluid changes marked by arrows. Both fibres equilibrated for 2 min in Ringer's solution with [Ca] of the following test solution.

The major effect of changes in $[Ca]_o$ was thus to change the level of $[K]_o$ at which contraction occurred and to influence the rate of relaxation in higher K-concentrations. The maximum tension developed in the fibres was very little affected by a change from 2 to 10 mM-Ca. In 0.2 mM-Ca the peak tension was somewhat lower in the high $[K]_o$-range. Thus, although the contractures in the slow fibres had a time course very different from that of the twitch fibres and a markedly different peak tension-$[K]_o$ dependence, the effects of changes in $[Ca]_o$ were quite similar (*cf.* Lüttgau 1963; Frankenhaeuser and Lännergren 1967).

The effect of altered [*Ca*]*o* *on the relation between* [*K*]*o* *and inactivation of the contractile system*

In a previous paper (Frankenhaeuser and Lännergren 1967) the term inactivation of the contractile system was introduced. Inactivation was there defined as the decrease in available tension, seen after conditioning a fibre with a solution of increased K-concentration. Available tension was tested by the application of a solution of 190 mM-K at the end of the conditioning period. The same procedure was adopted in corresponding experiments on slow fibres. The conditioning period was 1 min. Fig. 3 *gives the results from two consecutive experiments on two different fibres*. Fig. 3 *B* gives the values obtained in 2 mM-Ca. In this Ca-concentration a slow fibre gave a large maintained contracture in 20 mM-K. Application of 190 mM-K after 1 min resulted in a very small increase in tension followed by relaxation. In 40 mM-K there was a considerable decay in tension during a 1 min period. A change to 190 mM-K at the end of this did not lead to an increase in tension but only to a faster tension fall. This effect is different from that observed in twitch fibres. In the latter (see Frankenhaeuser and Lännergren 1967, Fig. 5 *B*) it was always possible to induce a second contracture by the application of 190 mM-K, provided the K-concentration in the conditioning solution was not too high (less than 60 mM-K).

(c) The action of altered $[Ca]_o$ is quite rapid in both types of fibre and can be seen within 1 sec in the twitch fibres and within 1—2 sec in the slow fibres. The action of altered $[K]_o$ is more rapid than that of altered $[Ca]_o$ in twitch fibres in slow fibres the two effects seem to set in simultaneously

A generally accepted view of the events leading to contraction in a twitch muscle fibre is the following Depolarization of the surface membrane spreads into the transverse tubules (T tubules) of the sarcotubular system and causes a release of an activating substance, probably Ca^{++} or a Ca-complex, from the lateral sacs, which in turn activates the neighbouring myofibrils (see reviews by Huxley 1964 Sandow 1965)

A tentative explanation for the differences in the dependence of tension on membrane potential in twitch and slow fibres may be given on the basis of the following assumptions (1) The release of the substance activating the contractile system is controlled by sites which become active at a certain level of potential, possibly but not necessarily identical in twitch and slow fibres. (2) These sites are localized in the central elements of the triads, the T tubules, in twitch fibres and close to the surface membrane of slow fibres.

Page (1965) has shown that the arrangement of the sarcoplasmic reticulum is different in the two fibre types, triads being very rarely found in slow fibres. The assumption that the activating sites are located close to the surface membrane of slow fibres finds support in the findings of Peachey and Huxley (1960) They noted a difference between twitch and slow fibres in the response to local electrical stimulation. Twitch fibres had sensitive spots only at the I-bands and activation spread radially inward as far as 10 µm without affecting adjacent sarcomeres. Slow fibres had sensitive spots both in A bands and I bands and activation spread as far longitudinally as radially often affecting three I-bands.

If the above assumptions are correct, the gradation of tension with external potassium concentration in twitch fibres would result from a spatial distribution of activated sites in the T tubules, deeper sites being activated only by a large $[K]_o$. In the slow fibres the sites would be activated in a more uniform manner thereby explaining the all-or none like contractures observed experimentally

If it be further assumed that calcium, by local action, controls the potential level at which the sites become active, this would explain why a change in $[Ca]_o$ acts more slowly upon a twitch fibre than does a change in $[K]_o$. In a twitch fibre it would take some time for calcium to diffuse into the T tubules, whereas in a slow fibre calcium would act at the same place as potassium, i.e. most likely at the surface membrane.

The effect of calcium on the tension — log $[K]_o$ relation is similar to that on the specific ion permeabilities in excitable membranes (Frankenhaeuser 1957 Frankenhaeuser and Hodgkin 1957) However the effect is qualitatively the same in twitch and slow fibres. Since slow fibres do not have a regenerative membrane, this means that the effect of calcium is not necessarily linked with the excitation process.

The assumptions above are also consistent with the findings in the inactivation

experiments. In the twitch fibres the first contracture seen during the initial concentration step may result from the activation of more peripherally located sites which become inactivated during the step. The next, larger step would then lead to a release of activator from more centrally located sites, causing a second contracture. In the slow fibres all sites would be activated and inactivated more or less simultaneously and hence no second increase in tension could be obtained experimentally.

It should be pointed out that the explanations given above for the difference in behaviour between twitch and slow fibres is based on assumptions which have not yet been tested. Although there is some independent evidence in favour of the hypothesis presented — the difference in fine structure (Page) and the outcome of the local activation experiments (Peachey and Huxley) — other possibilities are not excluded.

Since this manuscript was written, an article by G. A. Nasledov, J. Zachar and D. Zacharová "The ionic requirements for the development of contracture in isolated slow muscle fibres of the frog" *Physiol. bohemoslov.* 1966. *15*, 293—306, has appeared. These authors also found a latency in the onset of contracture. The relaxation in very high $[K]_o$ was not as pronounced as reported here. The authors also saw a shift of the peak tension — log $[K]_o$ curve with change in $[Ca]_o$ that agrees with the present findings.

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(c) The action of altered $[Ca]_o$ is quite rapid in both types of fibre and can be seen within 1 sec in the twitch fibres and within 1—2 sec in the slow fibres. The action of altered $[K]_o$ is more rapid than that of altered $[Ca]_o$ in twitch fibres in slow fibres the two effects seem to set in simultaneously

A generally accepted view of the events leading to contraction in a twitch muscle fibre is the following Depolarization of the surface membrane spreads into the transverse tubules (T tubules) of the sarcotubular system and causes a release of an activating substance probably Ca or a Ca-complex, from the lateral sacs, which in turn activates the neighbouring myofibrils (see reviews by Huxley 1964 Sandow 1965)

A tentative explanation for the differences in the dependence of tension on membrane potential in twitch and slow fibres may be given on the basis of the following assumptions (1) The release of the substance activating the contractile system is controlled by sites which become active at a certain level of potential possibly but not necessarily identical in twitch and slow fibres (2) These sites are localized in the central elements of the triads, the T-tubules, in twitch fibres and close to the surface membrane of slow fibres.

Page (1965) has shown that the arrangement of the sarcoplasmic reticulum is different in the two fibre types triads being very rarely found in slow fibres. The assumption that the activating sites are located close to the surface membrane of slow fibres finds support in the findings of Peachey and Huxley (1960) They noted a difference between twitch and slow fibres in the response to local electrical stimulation Twitch fibres had sensitive spots only at the I bands and activation spread radially inward as far as 10 μm without affecting adjacent sarcomeres. Slow fibres had sensitive spots both in A bands and I bands and activation spread as far longitudinally as radially often affecting three I-bands.

If the above assumptions are correct, the gradation of tension with external potassium concentration in twitch fibres would result from a spatial distribution of activated sites in the T-tubules, deeper sites being activated only by a large $[K]_o$. In the slow fibres the sites would be activated in a more uniform manner thereby explaining the all-or-none like contractures observed experimentally

If it be further assumed that calcium, by local action, controls the potential level at which the sites become active, this would explain why a change in $[Ca]_o$ acts more slowly upon a twitch fibre than does a change in $[K]_o$. In a twitch fibre it would take some time for calcium to diffuse into the T tubules whereas in a slow fibre calcium would act at the same place as potassium, i.e. most likely at the surface membrane.

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The assumptions above are also consistent with the findings in the inactivation

capillary pressure. These adjustments are often such that it may be questioned whether they do not, by some mechanism, influence the transport *per se*. It is true that in many situations it is easy to visualize the efficacy of this vascular response pattern from a hemodynamic point of view. For example in hemorrhage the reflex constriction leading to absorption of extravascular fluid into the circulatory system tends to restore the plasma volume (*e.g.* Folkow and Mellander 1964). In some situations, however, the beneficial effects of this particular vascular response pattern are less obvious. Vasodilatation is generally associated with a sufficient decrease in the ratio of pre- to postcapillary resistance to cause net filtration from plasma to tissues. This occurs, for example, during increased tissue metabolism, muscle exercise, engagement of the sympathetic vasodilator fibres and following administration of various vasodilator agents (for ref. see Folkow and Mellander 1964, Kjellmer 1965). Hemodynamically, a considerable transcapillary loss of fluid from the circulatory system would appear to be irrational in such situations, some of which call for an increased transport of nutrients and other solutes. If, on the other hand, by some mechanism, an increased transcapillary filtration could facilitate the transport of materials from blood to tissue, or *vice versa*, the net effect of such a vascular response might indeed be beneficial.

In the present study an attempt was made to test this hypothesis. For this purpose the rate of transport of materials from tissue to blood was studied in terms of tissue clearance at various levels of net filtration and net absorption. Demonstration of any such facilitated exchange requires that other factors influencing transport be kept as constant as possible, such as blood flow, flow velocity, capillary flow distribution, size of capillary surface area and capillary permeability. It is believed that with the experimental approach of the present investigation it was possible to control, directly or indirectly, these factors.

In biological systems the influence of net transcapillary fluid movement on solute transfer has been studied experimentally in only few investigations. Hyman *et al.* (1952) studied the clearance of isotopes from cutaneous tissue in humans during net transcapillary fluid movement produced by raising tissue protein osmotic pressure and concluded that tissue clearance is independent of capillary filtration. Since their experimental design did not allow complete control of all factors relevant to exchange, the general validity of their conclusion may be debatable.

Preliminary reports of this investigation have been published previously (Mellander and Lundgren 1966, 1967).

Method

General principle. In most experiments the transport of solutes from skeletal muscle tissue to blood was measured in terms of tissue clearance of various isotopes. The magnitudes of the clearance constant (k) depicting the slope of the disappearance curve for the isotope, plotted semilogarithmically, were compared at various levels of net transcapillary filtration and absorption. But, as pointed out in the introduction, in the present study it was necessary to keep the aforementioned factors known to influence the clearance constant, as constant as possible. It was particularly important to prevent fluctuation of vascular smooth muscle tone since such alterations indirectly influence most of the factors controlling capillary exchange. Variation in vascular tone was prevented

by denerv ting the region studied nd by administering vasodilator gents which laxed completely th vascula smooth muscle. Despite experimentally induced hanges in transmural pressure blood flow was maintained at onstant level throughout ea h experiment by controlling the rterio-venous pressur gradient.

The pillary fil tion oefficient, CFC, (ml fluid filtered per min, 100 g tissue nd mm Hg t nscapillary hydrostatic pressure gradient, see Cobbold et al. 1963) was repeatedly determined in the experiment to insure that the pillary parameters wer steady during the rious experimental manoeuvres. Under uch standardized conditions the rat of transcapillary filtration nd absorption ould be varied without interfering significa tly with other factors of importance for exchange. Filtration was produced by increasing the mean hydrostatic capillary pressure nd bsorption by raising the plasma osmotic pressure.

Operatic procedure. The animals were naesthetized l. with pentoba bital sod um (30—40 mg/kg). The study as performed on the skeletal muscles in the lower leg of the cat. The thigh muscles wer removed from the lower leg preparation by severing thei insertions nd the paw was removed t the nkle joint. The marrow ca ty of the femur was plugged ith cotton wool soaked in silicone grease. All vessels in th knee region except the popli eal artery nd vein ere ligated. The lower leg was placed i w ter filled, temperature ontrolled perspex plethysmograph to perm t continuous ecord ng of hanges in the tissue volume nd the skin on th lower leg, dissected free from the muscles, as used to seal the opening of the plethysmograph. Muscle blood flow as recorded w th silicone filled drop-recorder unit onnected to the popliteal vein. The arterial inflow was diverted from the proximal part of the femoral artery a short T-tube catheter t the popliteal rtery. This was done to permit close arterial administration of vasodilator drugs nd isotopes. Arterial inflow pressure was monitored from the femoral artery of the opposite limb and as adjusted by screw clamp placed around the bdominal orta. Venous outflow pressure was monitored from T-tube in the catheter diverting flow from the popliteal ein to the flowmeter. B screw lamp on his catheter venous pressure i he calf was djus ed to desired levels.

Isotope technique. Tissue blood transfer of ^{131}I, ^{24}Na, ^{86}Rb and ^{133}Xe as studied. I Na nd Rb are lipid-insoluble substances. I and Na ar distributed primarily t the terstitial compartment nd Rb to the intracellula ompartment. Xe is lipid-sol ble. The isotopes (N ^{131}I, ^{24}NaCl, ^{86}RbCl nd ^{133}Xe) were administered either by intramuscular injection (olume 0 1—0 15 ml) or usually by lose i tra arterial jection, or infusion lasting for 1—3 min. When I N and Rb were given i.a. enous blood from the region studied wa collected for about 3 to 5 min after the administration and this sample discarded to minimize recircula ion of isotope ma erial. This loss of blood was eplaced by equal mounts of Dextran Tyrode solution. Recirculation of ^{133}Xe was negligible, since it is almost complet ly eliminated from the blood on single passage through he lungs.

I most experiments the radiation from he region under study was recorded by an external scintillation detector fitted a wide angle lead collimator. Its sodium iodide crystal (1 3/4 2") was placed bove the muscle preparation outside the plethysmograph at distance of about 8 cm from the region. To record radiation from the muscles of the lower leg only other parts of the nimal were hielded by 10 mm or sometimes, 30 mm thick lead plate. I 10 expts. the disappearance of the isotopes (^{131}I ^{24}Na and ^{86}Rb) from the muscles fter initial tra-arterial infusion as followed by ontinuous recording of the radioactivity in the venous effluent. For his purpose venous ou flow was diverted coil of polyethylene tubing placed in well-type scintillation detector (P ckard 422).

The adioactivity was recorded in ll experiments with spectrometer (Auto-Gamma Model 410 A, Packard) connected to scaler and linea ratemeter (Model 280 A Packard) operating Honeywell or Rikadenki recorder. The time constant of the ra emeter was 3 or sometimes 10 sec. Pulses corresponding t gamma energies between 70 and 450 keV were counted for ^{131}I between 60 nd 200 keV for ^{133}X and bove 70 k V for ^{24}N and ^{86}Rb. The isotopes were administered in amounts sufficient to produce an initial count ra of leas 80,000 to 100 000 cpm. A continuous record from the ratemeter and intermittent counts for 0.5 or 1 min on the scaler were obtained. The recorded activity minus background was plotted semilogarithmically. The slopes of the learance curves were determined according to the method of leas squares.

I dealing with the transfer of ^{86}Rb from tissue to blood some special features deserve mentioning. After rapid vascula washout radioactivit y recorded by the external counter showed very slow decay probably due partly to omponent of slow transfer from the tracellular pool of Rb. A compartment analysis of the disappearance curve migh therefore be justified. The linear tail of the semilogarithmically plotted disappearance curve of ^{86}Rb was hen extrapolated to time zero nd subtracted from the original washout curve (*cf.* Dobson and Warner 1957).

Experimental procedure and calculation. Before administration of the isotopes he vascular bed was maximally dilated by slow continuous close arterial infusion of chloral hydrate (20—40 mg/min) or papaverine hydrochloride (abou 0.6 mg/min) in saline. That the dilatation was maximal as hecked by the finding that superimposed injection of large dose of acetylcholine produced no further increase in blood flow. Various rates of net filtration were produced by adjustments of

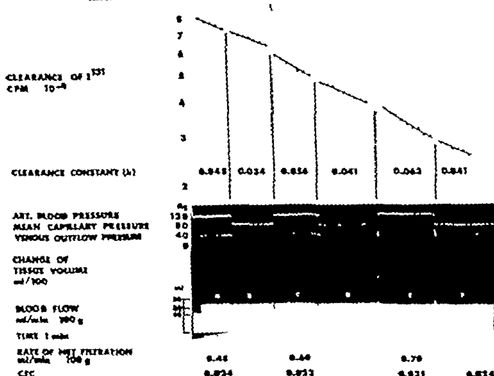


Fig. 1. Cat 3.5 kg. Nembutal. The effects of net transcapillary filtration on tissue clearance of ^{131}I (external counter) from skeletal muscle region. Vascular smooth muscle relaxed by i.a. infusion of papaverine. Note that clearance increased during the periods of filtration (A, C and E) compared with the control periods without net transcapillary fluid movement (B, D and F). Data of mean capillary pressure are calculated.

arterial inflow pressure and venous outflow pressure. Despite such changes in transmural pressure blood flow was kept constant (details shown in Fig. 1). Absorption was produced by slow infusion i.a. of 30 % glucose solution (in saline) or of 20 % plasma albumin solution (in saline).

The rate of filtration or absorption was calculated from the continuous incline or decline of the recorded tissue volume (Mellander 1960). The changes in mean hydrostatic capillary pressure were calculated according to Pappenheimer and Soto-Rivera (1948). This calculation required determination of the post- to precapillary resistance ratio, which was done according to Pappenheimer and Soto-Rivera (1948). During maximal dilatation this ratio was about 1 : 4. The change in plasma osmotic pressure during absorption was approximately estimated by dividing the recorded rate of fluid absorption by CFC. CFC was determined by raising venous outflow pressure a known amount (Pappenheimer and Soto-Rivera 1948). Details concerning the calculation of CFC have been described elsewhere (Cobbold *et al.* 1963, Mellander, Öberg and Odelram 1964).

Material. Experiments of the type described above were performed on 43 cats. ^{131}I was used in 19 of the animals, ^{85}Kr in 5, ^{86}Rb in 16 and ^{133}Xe in 3. In another series of experiments performed on 7 cats the pressure gradient was not held constant and hence blood flow varied. Results, section C.

Results

A. *Effects of net transcapillary fluid movement on tissue-blood transfer of isotopes measured by external counter*

a. ^{131}*Iodide.* Fig. 1 shows the rate of disappearance (tissue clearance) of ^{131}I from the muscles of the lower leg during transcapillary net filtration at various rates. A pronounced and stable relaxation of the vascular smooth muscle was obtained

by denervating the region studied and by administering vasodilator agents which relaxed completely the vascular smooth muscle. Despite experimentally induced changes in transmural pressure, blood flow was maintained at a constant level throughout each experiment by controlling the arterio-venous pressure gradient.

The capillary filtration coefficient, CFC, (ml fluid filtered per min/100 g tissue and mm Hg transcapillary hydrostatic pressure gradient, see Cobbold et al. 1963) was repeatedly determined in the experiment to insure that the capillary parameters were steady during the various experimental manoeuvres. Under such standardized conditions the rate of transcapillary filtration and absorption could be varied without interfering significantly with other factors of importance for exchange. Filtration was produced by increasing the mean hydrostatic capillary pressure and absorption by raising the plasma osmotic pressure.

Operative procedures. The animals were anaesthetized i.v. with pentobarbital sodium (30—40 mg/kg). The study was performed on the skeletal muscles in the lower leg of the cat. The thigh muscles were removed from the lower leg preparation by severing their insertions and the paw was removed at the ankle joint. The marrow cavity of the femur was plugged with cotton wool soaked in silicone grease. All vessels at the knee region except the popliteal artery and vein were ligated. The lower leg was placed in a water filled, temperature controlled perspex plethysmograph to permit continuous recording of changes in the tissue volume and the skin on the lower leg, dissected free from the muscles, was used to seal the opening of the plethysmograph. Muscle blood flow was recorded with a silicone-filled drop-recorder unit connected to the popliteal vein. The arterial inflow was diverted from the proximal part of the femoral artery via a short T-tube catheter to the popliteal artery. *This was done to permit close arterial administration of vasodilator drugs and isotopes.* Arterial inflow pressure was monitored from the femoral artery of the opposite limb and was adjusted by a screw clamp placed around the abdominal aorta. Venous outflow pressure was monitored from a T-tube in the catheter diverting flow from the popliteal vein to the flowmeter. By a screw clamp on this catheter venous pressure in the calf was adjusted to desired levels.

Isotope technique. Tissue-blood transfer of ^{131}I, ^{24}Na, ^{86}Rb and ^{133}Xe was studied. I, Na and Rb are lipid-insoluble substances, I and Na are distributed primarily to the interstitial compartment and Rb to the intracellular compartment. Xe is lipid-soluble. The isotopes (Na^{131}I, ^{24}NaCl, ^{86}RbCl and ^{133}Xe) were administered either by intramuscular injection (volume 0.1—0.15 ml) or, usually, by close intra-arterial injection, or infusion lasting for 1—3 min. When I, Na and Rb were given i.a., venous blood from the region studied was collected for about 3 to 5 min after the administration and this sample discarded to minimize recirculation of isotope material. This loss of blood was replaced by equal amounts of Dextran-Tyrode solution. Recirculation of ^{133}Xe was negligible since it is almost completely eliminated from the blood on a single passage through the lungs.

In most experiments the radiation from the region under study was recorded by an external scintillation detector fitted in a wide angle lead collimator. Its sodium iodide crystal (1 3/4 × 2") was placed above the muscle preparation outside the plethysmograph at a distance of about 8 cm from the region. To record radiation from the muscles of the lower leg only, other parts of the animal were shielded by 10 mm or sometimes 30 mm thick lead plate. In 10 expts. the disappearance of the isotopes (^{131}I, ^{24}Na and ^{86}Rb) from the muscles after initial intra-arterial infusion was followed by continuous recording of the radioactivity in the venous effluent. For this purpose venous outflow was diverted to a coil of polyethylene tubing placed in a well-type scintillation detector (Packard 422).

The radioactivity was recorded in all experiments with a spectrometer (Auto-Gamma Model 410 A, Packard) connected to a scaler and a linear ratemeter (Model 280 A, Packard) operating a Honeywell or Rikadenki recorder. The time constant of the ratemeter was 3 or sometimes 10 sec. Photons corresponding to gamma energies between 70 and 450 keV were counted for ^{131}I, between 60 and 200 keV for ^{133}Xe and above 70 keV for ^{24}Na and ^{86}Rb. The isotopes were administered in amounts sufficient to produce an initial count rate of at least 80,000 to 100 000 cpm. A continuous record from the ratemeter and intermittent counts for 0.5 or 1 min on the scaler were obtained. The recorded activity minus background was plotted semilogarithmically. The slopes of the clearance curves were determined according to the method of least squares.

In dealing with the transfer of ^{86}Rb from tissue to blood some special features deserve mentioning. After a rapid vascular washout, radioactivity recorded by the external counter showed very slow decay probably due partly to a component of slow transfer from the intracellular pool of Rb. A compartment analysis of the disappearance curve might therefore be justified. The linear tail of the semilogarithmically plotted disappearance curve of ^{86}Rb was then extrapolated to time zero and subtracted from the original washout curve (*cf.* Dobson and Warner 1957).

Experimental procedures and calculations. Before administration of the isotopes the vascular bed was maximally dilated by a slow continuous close arterial infusion of chloral hydrate (20—40 mg/min) or papaverine hydrochloride (about 0.6 mg/min) in saline. That the dilatation was maximal was checked by the finding that a superimposed injection of a large dose of acetycholine produced no further increase in blood flow. Various rates of net filtration were produced by adjustments of

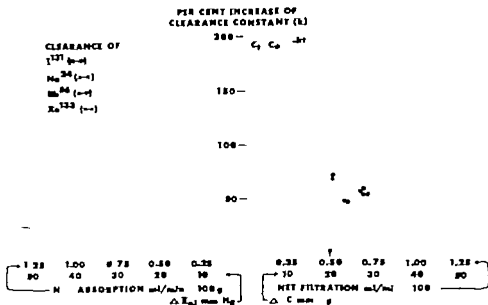


Fig 3 Collected data to show the increase in clearance constants (k) of ¹³¹I ⁸⁶Rb, ²⁴Na and ¹³³Xe during various rates of transcapillary fluid movement (filtration right side, absorption left side) The approximate change of mean hydrostatic capillary pressure (Δ pC) and plasma osmotic pressure (Δ pl) are also indicated. The increase of k during net transcapillary fluid movement are expressed in per cent of the control values of k obtained when no net transcapillary fluid movement occurred.

tissue. CFC, reflecting the capillary porosity and the size of the capillary surface area available for exchange was found to be virtually the same during high and low transmural pressures (Fig 1)

The disappearance curve for ¹³¹I plotted semilogarithmically is shown in the upper panel, from which it is clear that the curve is linear during each experimental procedure. Its slope is, however greater during high than during low transmural pressure, indicating an increased rate of disappearance of ¹³¹I during the periods of net filtration. The magnitude of this change is more clearly revealed by comparing the clearance constants (k) depicting the slopes of the various disappearance curves (Fig 1) During the first period with prevailing net filtration (A) tissue clearance was about 40 per cent higher than during the following control period without filtration (B) In section C, where filtration was somewhat greater than in A, tissue clearance exceeded the control value (mean of B and D) by about 45 per cent. In E, tissue clearance was about 55 per cent above the control values (D F)

It may be argued that the changes in disappearance rate observed for fairly short time, as in Fig 1 reflect transient changes which are not necessarily representative of the steady state Similar experimental manoeuvres prolonged for 5 to 15 min clearly demonstrated that the increase in clearance constant during manifest net filtration as compared with the control value was not transient but constantly maintained Fig 2

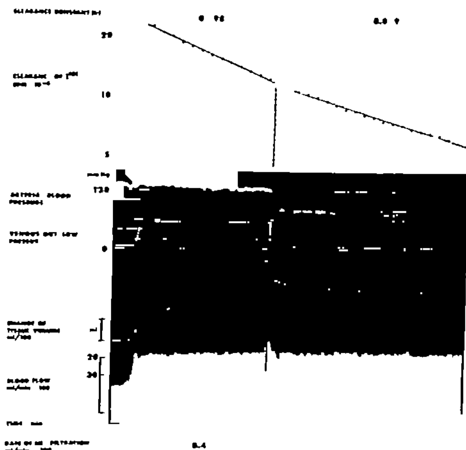


Fig 2 Cat 3.8 kg Nembutal. Similar type of experiment as shown in Fig 1 but with longer periods of observation. CFC was found to be constant during high and low transmural pressure

throughout the course of the experiment by continuous close intra-arterial infusion of papaverine (about 1 mg/min × 100 g tissue) With this, the blood flow was around 40 ml/min × 100 g tissue at a normal pressure gradient. In A venous outflow pressure was raised about 30 mm Hg which resulted in a transcapillary filtration of 0 48 ml/min × 100 g tissue (continuous, slow increase of tissue volume cf Pappenheimer and Soto-Rivera 1948, Mellander 1960) The calculated rise in mean hydrostatic capillary pressure was about 20 mm Hg Blood flow decreased to about 25 ml/min × 100 g tissue owing to the decrease of arterio-venous pressure gradient. The blood flow was then maintained constant throughout the experiment. In B, both arterial inflow pressure and venous outflow pressure were lowered, thus reducing mean capillary pressure so that net filtration stopped The intravascular pressures were then again raised (C) so as to produce a net filtration of 0 60 ml/min × 100 g tissue, after which the pressures were again lowered (D) Similar experimental manoeuvres were done in E and F In E, the net filtration was 0 70 ml/min × 100 g

filtration was associated with an increase of clearance constant above control level. The data presented here as well as those described below include observations following administration of isotope by intramuscular injection and by close i.a. injection or infusion. It should be emphasized that no consistent difference in effects obtained could be ascribed to the use of one or the other of these two routes of administration.

In another series of experiments the effects of net transcapillary absorption of extravascular fluid on the disappearance rate of ^{131}I were investigated. Fig 4 demonstrates such an experiment in which absorption was produced by a slow close arterial infusion of a 30 glucose solution (signal) The average absorption rate in this period (before the CFC determination) was about 0.6 ml/min × 100 g tissue. By crude approximation it could be calculated that the glucose infusion produced an *average transcapillary osmotic pressure gradient of about 20 mm Hg* The clearance constant of ^{131}I increased by about 75 per cent above control level during the period of extravascular fluid absorption. On cessation of the glucose infusion fluid returned from blood to the tissue this was evidently due to establishment of a reversed transcapillary osmotic gradient The rate of net transcapillary movement of fluid into the tissue was about 0.3 ml/min × 100 g tissue The clearance constant exceeded the control values by about 35 per cent in this period. Five to 6 min later when the net transcapillary fluid movement had virtually ceased, the clearance constant returned approximately to the initial control level (Fig 4 right panel) The CFC remained practically constant during the whole course of this experiment Fig 4) The demonstration that the clearance constant increased during the above mentioned phase of fluid return to the tissue deserves mentioning since it shows that outward transcapillary fluid movement facilitates transport whether the driving force is hydrostatic or osmotic. Results similar to those described above were obtained in experiments where absorption was produced by infusion of a 20 plasma albumin solution Transcapillary absorption of fluid was invariably associated with an increase in clearance constant of ^{131}I above control level Fig 3 left side open circles Further *fluid absorption seemed to be as effective in increasing the disappearance rate of* ^{131}I from tissue to blood as was net filtration

b Rubidium. Experiments similar to those described so far were performed after loading the muscles with the tracer ^{86}Rb After an initial rapid vascular wash-out disappearance occurred at a very slow rate The clearance constant was about 1/10 to 1/20 of that of ^{131}I under the same control conditions Since the tissue blood partition coefficient for Rb is in the range of 20 the clearance rate of Rb should be of about the same magnitude as that of ^{131}I This slow disappearance curve showed increased slopes during periods of net filtration or absorption as compared with control states in the way described above for ^{131}I It is evident that for such low disappearance curves, even a great increase in slope would correspond to only quite small absolute changes in recorded radioactivity A quantitative evaluation of such changes might involve a certain degree of inaccuracy By compartment analysis of the disappearance curve as described in Methods, a fast component of wash-out

of Rb was obtained with a clearance constant of about the same magnitude as that for ^{131}I. This component constantly showed increased k-values during periods of net filtration in comparison with the control values, the magnitudes of which were easily quantitated. The results obtained with Rb in Fig. 3 (closed circles) represent data obtained after this compartment analysis. It can be seen that the clearance constant for ^{86}Rb is increased above control level both during net filtration (right panel) and absorption (left panel). Although the range of variation is fairly wide most of the Rb-values are close to those obtained for ^{131}I.

c. Sodium. The disappearance rate of Na was of about the same order of magnitude as that of ^{131}I under the same control conditions. The clearance constants of Na during the control state of zero net filtration and during states of graded net filtration were compared in the way described above for ^{131}I. The effects of absorption were not investigated. As is evident from Fig. 3 which summarizes the results (x—x) the clearance constant increased during net filtration above control. The effects obtained for Na seem to be of similar magnitudes as those found for ^{131}I.

d. ^{133}Xenon. The disappearance rate of ^{133}Xe was found to be about 2 to 3 times as great as that of ^{131}I under the same control conditions within the range of blood flows studied. As for Na, the disapperance rate was followed during net filtration. Fig. 3 summarizes the results (squares). It can be seen that the clearance constant increased above control level during filtration but the percentage change appears to be less than for the three water-solubl isotopes tested. It was often found that the disappearance curve of ^{133}Xe under control conditions was not strictly mono-exponential, but the curve plotted semilogarithmically showed a clearance constant gradually decreasing with time. This made it more difficult to reveal the effects of filtration on the wash-out of Xenon than on wash-out of the other isotopes which generally showed a less marked deviation from mono-exponential decay. The diagram includes only data from experiments where filtration increased the clearance constant for ^{133}Xe above that in a *preceding* control period. Without filtration the clearance constant showed a decrease with time, but the size of the decrease was difficult to judge exactly. The control clearance constant was taken as a mean of values obtained before, and immediately after the period of filtration. For these reasons the data on Xenon given in Fig. 3 can only be considered approximate.

B. Effects of net transcapillary filtration on tissue-blood transfer of isotopes measured as changes in venous radioactivity

The transfer of ^{24}Na, ^{131}I and Rb from tissue to blood was investigated by monitoring venous radioactivity after an initial period during which the muscle had been loaded with tracers by intra-arterial administration. In several of these experiments the disappearance rates were recorded simultaneously by an external counter. In the control state of zero net filtration there was a continuous decline in recorded venous radioactivity. Transition from the control state to a state of induced net filtration was almost without exception associated with a considerable increase in venous radio-

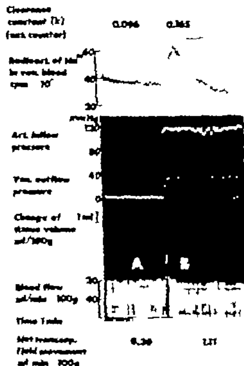


Fig 3. Transfer of ^{24}Na from skeletal muscle tissue to blood measured simultaneously by external counter (clearance constant) and by following changes in venous radioactivity (cpm per cent). After transition from state of only slight net filtration (A) to state of considerable net filtration (B) the clearance constant increased by about 75 per cent and, simultaneously, venous radioactivity increased by about 85 per cent. Blood flow and CFC virtually the same in A and B. For further details see text.

activity The radioactivity reached a peak value within 10 to 30 sec after the transition, after which the activity again gradually declined.

Fig 3 shows this effect observed in an experiment with Na. In section A with low transmural pressure there was a slight net filtration 0 3 ml min 100 g tissue) CFC was determined earlier in this period and was found to be 0 029 ml/min mm Hg × 100 g tissue In B transmural pressure was raised so as to produce a considerable net filtration (1 11 ml/min × 100 g tissue or 0 81 ml min more than in A) without significantly changing the regional blood flow CFC was then 0 028 ml/min × mm Hg × 100 g The upper curve shows the continuous record of venous radioactivity Upon transition from low to high transmural pressure the activity increased from about 33,000 to 62,000 cpm, or roughly by 85 per cent The clearance constants during these two periods derived from the disappearance curves recorded by an external counter are shown at the top of Fig 3 During high transmural pressure B) there was an increase in clearance constant of bout 75 per cent above that in the preceding period A This experiment thus shows an increased transfer of Na from tissue to blood in B, the increase being approximately the same whether it was computed from the recorded increase in venous radioactivity or from the change in clearance constant determined by external counter Since the amount of the tracer in the tissue is far from infinite an increased disappearance will be observed not only as a rapidly decreasing radioactivity recorded externally but also, sooner or later as decreasing radioactivity in the venous blood. This implies that

during the whole period of increased filtration there will not be a constantly maintained high level of venous radioactivity but, after the peak, a fairly rapid decline (Fig. 5).

In analogy with the above results all tracers studied showed a decrease in venous radioactivity on return from states of net filtration to control states. This change was, as a rule, not so abrupt and sometimes not so pronounced as the corresponding increase during transition from low to high transmural pressure.

C. Effects of transcapillary filtration on tissue-blood transfer of isotopes during reduced blood flow

It is well known that a relation exists between the rate of tissue clearance and the magnitude of blood flow. As far as lipid-soluble substances are concerned such a relationship appears to prevail over a very wide range of blood flows. For lipid-insoluble substances it seems to be clearly demonstrable only in the lower flow range (see below). In the present study an attempt was made to determine whether the decrease of the clearance constant associated with a decrease in blood flow could be at least partly compensated for by a superimposed net filtration (Fig. 6). In this experiment the disappearance of ^{131}I was studied (external counter). Blood flow

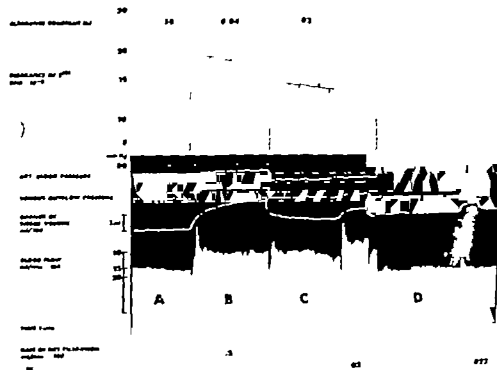


Fig. 6. Cat 3.4 kg. Nembutal. Tissue clearance of ^{131}I during various rates of blood flow and net transcapillary fluid movement. Vascular smooth muscle relaxed by i.a. infusion of papaverine. Clearance curves dr[illegible] ording [illegible] the method of least squares. For description see text.

was 40 ml/min × 100 g tissue in this maximally dilated skeletal muscle at normal perfusion pressure (not shown in Fig 6) In section A of the figure the arterial inflow pressure was lowered so as to reduce blood flow to the range in which clearance of ^{131}I was clearly flow dependent (about 15 ml/min × 100 g tissue) Venous outflow pressure was adjusted in such a way that no net transcapillary fluid movement occurred. In B and C, blood flow was further reduced to an approximately constant level of about 10 ml/min × 100 g tissue. In B, this was accomplished by raising venous outflow pressure so that the calculated mean capillary pressure rose by about 15 mm Hg which, in turn, produced a net filtration of 0.27 ml/min × 100 g In C, on the other hand, the flow reduction was maintained by lowering arterial inflow pressure and venous outflow pressure, keeping the arterio-venous pressure gradient approximately constant. The calculated mean capillary pressure fell by about 20 mm Hg and net filtration stopped. In D finally arterial inflow pressure was again increased so that the blood flow returned approximately to the control level of A. CFC was virtually constant during the various experimental steps (see Fig) In the control periods of A and D (large blood flow no net filtration) the clearance constant of ^{131}I was 0 058 and 0 055 respectively A reduction of blood flow without concomitant net filtration (C) was associated with a considerable decrease of the clearance constant (k = 0.027) The same degree of flow reduction in the face of a net filtration (B) on the other hand, was associated with a much smaller decrease of the clearance constant (k = 0.048) It would therefore appear that the presence of a net transcapillary movement of fluid can improve considerably tissue-blood transfer in situations of decreasing blood flows. This conclusion was arrived at in all experiments of this type performed with ^{131}I as well as in similar ones using ^{86}Rb

Discussion

In current discussions concerning the exchange of materials between the blood and tissue interest has been focused mainly upon such variables as blood flow flow velocity capillary flow distribution, capillary surface area, capillary permeability and the length of the diffusion distances between blood and tissue cells (Kety 1951 Pappenheimer 1953 Landis and Pappenheimer 1963) The present study suggests that an additional factor deserves consideration viz., the net transcapillary movement of fluid It was shown in this study that in skeletal muscle the presence of a net transcapillary fluid filtration or absorption *per se* increased the rate of transfer of ^{131}I Rb, Na and ^{133}Xe from tissue to blood above the level existing during Starling equilibrium, when no net fluid movement occurs (for Na and Xe only filtration studied) Within a certain range, there was a progressive increase in transfer with increase in rate of transcapillary fluid movement at least with regard to the water soluble substances (Fig 3)

A demonstration of transcapillary fluid movement as an independent variable influencing exchange requires control of other factors of importance. It is believed that with the experimental approach used (except that in Results, section C) t

interference of these other factors was insignificant This assumption is briefly discussed below

The study was performed on a denervated and sympathectomized skeletal muscle region dilated to or close to maximum. This served several purposes It secured a pronounced and stable relaxation of the vascular smooth muscle and prevented changes in vasomotor tone. This was of particular importance in this type of experiments where great changes of transmural pressure were sometimes produced. It is known that an increase in transmural pressure tends to constrict both resistance vessels and precapillary sphincters even in a denervated region (Folkow 1962 Mellander Öberg and Odelram 1964) this, in turn would affect virtually all of the above mentioned factors of importance for exchange. In the present nonreactive vascular bed it is much easier to secure a constant blood flow capillary flow distribution etc. Further by abolition of the vasomotor tone blood flow was increased to the range (above 15 ml/min × 100 g) in which the clearance of ^{131}I Na and ^{86}Rb is assumed to be comparatively little flow dependent (*cf* Renkin 1959 Landis and Pappenheimer 1963 Lassen 1964)

Another important technical detail in this study is the avoidance of a constant-flow pump placed on the arterial side Experience has shown that rough handling of arterial blood by peristaltic pumps etc. sometimes causes aggregation of thrombocytes etc., which can then plug capillaries and produce gradual or sudden changes in the size of the capillary exchange surface area Flow measurements on the venous side interfere little with the regional circulation since aggregates, if produced, are trapped in the pulmonary vascular bed

CFC is a *direct measure of transcapillary hydrodynamic conductivity and provides* an indirect control of two of the above mentioned factors, namely capillary surface area available for exchange and capillary permeability" In fact, it gives information about the product of these two factors as regards permeation of water and evidently of several other substances as well. This conclusion is based on the consistent results obtained with this method and with quite independent methods the extent to which this product can vary under different physiological conditions was found to be largely the same by all these techniques (Renkin and Rosell 1962, a, b Cobbold *et al* 1963 Wright and Sonnenschein 1965 Dresel, Folkow and Wallentin 1966) Since under the various experimental manoeuvres of this study CFC was found to be insignificantly changed the conclusion can be drawn that capillary surface area and capillary permeability were not significantly altered Further since blood flow was kept constant, the finding of an unchanged CFC indicates a constant capillary flow distribution and a virtually constant capillary blood flow velocity It should also be emphasized that the constancy of CFC during low and high transmural pressures seems to rule out the possibility of a stretched pore phenomenon (Shirley *et al* 1957) during high pressure Our findings thus agree with previous observations of Landis and Gibbon (1933) and of Grotte (1956) Further this observation showed that a passive closure of capillaries did not occur in the present study during low hydrostatic pressures nor was this expected since the transmural

pressure levels were always kept well above so-called critical closing" levels (Nichol et al 1951) The results obtained cannot possibly be attributed to simple hydrostatic distention of the vascular bed, since increased tissue-blood transfer of solutes was also observed during transcapillary absorption and during net movement of fluid from blood to tissue produced by osmotic forces in the face of unchanged hydrostatic pressure (Fig 4)

The volume of the interstitial fluid compartment varied to some extent during these experiments and, with it, probably also the diffusion distances between blood and tissue. Thus, during filtration these distances might increase to some extent owing to accumulation of extravascular fluid Despite this, transfer of solutes was found to increase during filtration During absorption the diffusion distances might decrease gradually It should be noted, however that, in all experiments done, the semilogarithmic clearance curves seemed to be linear during the whole period of absorption and did not show a gradually ncreasing deflection as would be expected with progressively decreasing diffusion distances. Therefore, the results observed can ot possibly be attributed to changing lengths of the diffusion pathways. Finally with the techn que used here, the partition coefficient must be taken into account (Kety 1951) It seems unlikely however that the experimental manoeuvres of the present study would instantaneously produce such great cha ges in the partition coefficient as to account for the results obtained

From this discussion it seems justified to conclude that the ariable responsible for the observed increase in clearance rate is related to the net transcapillary fluid movement *per se*

Although an analysis of the ultimate mechanism by which transcapillary fluid movement enhances tissue-blood transfer is beyond the scope of the present investiga tion few *speculative* comments may be justified The facilitation of solute transfer might not necessarily occur across the capillary membranes, but rather in the inter stitial fluid compartment. This compartment, believed to be built up of a dense meshwork of fibrils and/or long molecular chains n a gel phase fluid (*cf* Laurent 1966) might offer considerable resistance to ionic distribution, perhaps, much more than the capillary membranes themsel es (cf Landis and Pappenheimer 1963 p. 1023) A non uniform distribution of molecules might therefore frequently exist in this space and t is possible that the produced transcapillary movement of fluid facilitates, by convection and/or convection-diffusion, the transport of solutes within the interstitial compartment. Hydrostatic gradients between adjacent capillary loops are probably quite common, so that transcapillary fluid movements might produce both paracapillary and ntercapillary streaming in the interstitial space. Further during filtration the filtered fluid may remain, at least temporarily in a sol phase n the interstitial compartment where a gelatinous matrix is normally present. Since some data indicat that diffusion is more rapid in a sol than in gel phase (*cf* Jacobs 1935 Day 1952, Ogston and Sherman 1961) diffusion in the interstitial fluid might be facil tated during filtration. Other observations suggest that the inter stitial compartment contains a sy tem of canaliculi (*e g* McMaster and Parso

through which diffusion transport mainly occurs. If these pathways are distended during filtration transport may be facilitated Since, undoubtedly several other explanations are possible the question about the ultimate mechanism responsible for the present findings remains open

Although shown for only four substances so far there is reason to believe that the presence of an increased net transcapillary fluid movement will facilitate the transfer of other substances as well perhaps particularly lipid-insoluble agents. The influence of net transcapillary fluid movement on transport of material in the reverse direction from blood to tissue has not yet been studied It may not necessarily be taken for granted that this transport is facilitated in a similar way especially f the above mentioned mechanisms are involved.

It appears that the described factor might play a significant role in controlling tissue blood exchange in several physiological situations. As pointed out in the introduction most vascular reactions are associated with marked changes in the paracapillary circulation. During vasodilatation produced, for example, by increased tissue metabolism, the accompanying filtration may help suddenly to increase the rate of transport from the cells and may be a crucial factor n situations in which nutritional blood flow and capillary exchange surface area ha e approached their upper limits for increasing the exchange (*cf* Fig 6) Thus, the seemingly irrational transcapillary fluid loss from the circulation occurring in most states of vasodilatation might be considered as an adequate vascular response Similarly the absorption of extra vascular fluid occurring in skeletal muscle during vasoconstriction might be a factor tending to compensate for the decreased transport capacity secondary to the concomitant reduction in blood flow Further it seems possible that the para capillary circulation existing during a Starling equilibrium is one factor controlling rate of normal tissue-blood exchange although this has not been demonstrated y *in the present experiments. The facilitation of tissue clearance by changes* the paracapillary circulation may be a factor deserving attention when judging e validity of various clearance methods used for regional blood flow determination

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Acta physiol scand. 1967 70 42—53

From the Instit t for Medical Cell Research nd Genetics, Medic l Nobel Institute, Karolinska Instit tet Stockholm, Sweden

Mast Cells and 5-HT

Uptake of labelled 5-hydroxytryptamine (5-HT) and 5-hydroxytryptophan in relation to storage of 5-HT in individual rat mast cells

By

Martin Ritzén

Recei ed 15 November 1966

Abstract

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The capacity of individual rat peritoneal mast cells to take up and concentrate radioactively labelled 5-hydroxytryptamine (5-HT) and 5-hydroxytryptophan (5-HTP) was studied by quantitativ cytochemical methods By means of consecuti application of microfluorometric utoradiographic and microinterferometric methods, the following parameters could be determined for each cell 1 Relati mount of endogenous 5-HT 2. Concentration of accumulated isotopes. 3 Dry mass. 4 Concentration of endogenous 5-HT 5 Total mount of accumula ed isotopes The calculated correlations of these variables t each other showed general heterogeneity of the population, variations between the different cells being very large However on the whole increasing dry mass of mast cells was accompanied by increase in 5-HT content, but with decrease in concentra- ons of endogenous 5-HT and ccumula ed radioactive 5-HTP Similarly cells ha ing large stores endogenous 5-HT showed higher otal uptake, but lower cellula concentration of exogenous 5-HT and 5-HTP than cells tha were poor in endogenous 5-HT The results indicated that the ptake of H 5-HTP was proportional t the total rea of the cell membrane while the degree of 5-HT uptake was determined by other factors When the concentrations of radioactivit the incubation medium was compared t that of the living mast cell, it was found tha the erage cell was able t concentra exogenous 5-HTP and 5-HT round 4 and 30 times, respectively

For more than a decade 5-hydroxytryptamine (5-HT) has been recognised as a characteristic component of rat and mouse mast cells, but the precise role of this amine in the mast cell is still obscure Rat mast cells have been shown to possess the facilities to synthesize 5-HT by decarboxylation of the precursor amino acid 5-hydroxytryptophan (5-HTP) to yield 5 HT (Lagunoff and Benditt 1959) and to take up and concentrate both 5-HT and histamine (Furano and Green 1964)

Recently the histochemical fluorescence method of Falck and Hillarp for demonstration of biogenic monoamines has been utilised by a number of authors in order to identify the amine present in mast cells of various species and for studying the uptake of 5-HT its precursor amino acid or other amines and their precursors No

quantitative cytochemical data concerning the uptake of biogenic amines in mast cells have so far been published.

In a previous report, (Ritzén 1967a) it was shown, by combined model and mast cell experiments that the method of Falck and Hillarp can be used to get adequate measures of the *amount of 5-HT present in individual rat peritoneal mast cells*. It has also been shown (Ritzén *et al.* 1965 Hammarström *et al.* 1966) that after binding of the amines to the tissues by fixation with gaseous formaldehyde, radioactively labelled 5-hydroxytryptophan or 5-HT can preferably be localized by conventional autoradiographic procedures. It was then noted that ^3H 5-HTP accumulated in mouse peritoneal mast cells. In addition to earlier biochemical data concerning 5-HT uptake, storage and release in samples of more or less purified mast cell suspensions, this opens the possibilities to obtain information about these processes in the individual cells, without further isolation and purification procedures. The present investigation was undertaken in order to investigate the possible relations between uptake of trace amounts of exogenous 5-HT or 5-HTP and the level of endogenous 5-HT in the same peritoneal mast cell, and for a limited number of cells, to correlate these variables to the dry mass of the same cells. This can be achieved by consecutive applications of quantitative microfluorometric, autoradiographic and microinterferometric methods.

Material and methods

The following radioactive isotopes were used dl 5-hydroxytryptophan-T (G) (^3H 5-HTP) specific activity 3650 mc/mmole, 5-hydroxytryptamine creatinine sulphate-T (G) (^3H 5-HTP) 365 mc/mmole, and dl 3(3 4-dihydroxyphenyl)alanine-T(G) (^3H DOPA) 500 mc/mmole, all purchased from the Radiochemical Centre, Amersham, England. The radiochemical purity was reported by the manufactures to be >98 per cent. Before use, the tritium labelled amino acids were diluted with the respective non-radioactive compounds to give final specific activity of 365 mc/mmole equal to that of ^3H 5-HT

Mast cell preparations

In order to avoid variations between cells of different animals, only one male albino rat (strain R, obtained from Dr G. Bloom) weighing around 300 g and 6 months old, was used in each experiment. Six ml of buffered salt solution (Uvnäs and Thon 1959) containing 0.1 per cent bovine serum albumin (Armour) and heparin (10 IU/ml) was injected into the peritoneal cavity immediately after the rat being exsanguinated under light ether anesthesia. After gentle massage of the abdomen for 1 to 2 min the fluid was collected in an ice-cooled test tube and divided in 2 to 4 parts. The cells were gently centrifuged down and the supernatants discarded. The cells in each test tube were then resuspended in 5 ml Eagles medium (MEM) for tissue culture (Eagle 1965) to which bovine serum albumin and radioactive isotope had been added to concentrations of 0.1 per cent and 10 μc/ml, respectively. After incubation of the cell suspensions in open test tubes for 10 to 60 min at 37 C, the cells were gently centrifuged down, washed once with 10 ml buffered salt solution (including 0.1 per cent albumin) and recentrifuged. From the resulting cell sediment, brush preparations were made on microscopical slides as previously described (Ritzén 1967). The specimens were first dried in open air then kept for at least half hour in vacuum over phosphorous pentoxide. After completed drying the specimens were incubated for two hours at 80° C with paraformaldehyde that had been equilibrated with air of around 50 per cent relative humidity (Hamberger *et al.* 1965)

Microfluorometry

After examination in the fluorescence microscope coverslip was sealed with hot paraffi on the microscope slide without any mounting medium. Crystals resulting from the dried salt solution, that seriously impaired the microscopic image when viewed in ordinary transmitted light, were not visible in the fluorescence microscope. Maps of parts of the specimens were dra

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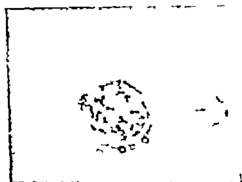


Fig. 2. Photomicrograph of ra peritoneal mast cell that has been incubated for one hour with H-3-HTP fixed in formaldehyde apours nd subjected to utoradiography The grain density over this cell corresponds to around 125 μc g cellular dry weight. The mast cell itself and the neighboring non-labelled whit blood cells are in another focus than the photographic grains. Phase contrast, magnification 1,200 ×

Microinterferometry

After the grain density over the cells had been determined, the photographic emulsion was dissol ed in hot water (70°C) for 30 to 60 min. Although this treatment completely dissol ed the emulsion, a number of photographic grains were left on the cells. These black grains, which would seriously hav ffected the interference measurements, were bleached and dissolved by immersion in 0.5 per cent solution of potassium bichromate in 1 per cent sulphuric acid for 2 min, followed by rising in distilled water for 30 min. After this, the specimens were gain mounted for microscopy in glycerin. The relative dry mass of the cells that previously had been investigated with respect to fluorescence and radioactivity was then determined by means of rapid scanning microinterferometer (Caspersson and Lomakka 1962, Lomakka 1965) No significant difference in dry mass was found between the mast cells tha had undergone the autoradiographic procedure (including dissolution of the film) and 25 other mast cells from the sample, hat were measured in gly erin directly after formaldehyde apour fixation.

Unfortunately it proved to be necessary to perform the autoradiographic procedure before the interferometric measurements since the mounting medium, necessary for the latter measurements, dissolved part of the radioactiv compounds from the cells. This fact necessitated the rather laborious and somewha hazardous (the cells migh burst) remov al of the emulsion and remaining grains after the grain countings had been completed Although the 5-HT in the mast cells was most probabl dissol ed during this treatment, the relativ dry mass still was good measure of the size of the cell.

The concentration of solids in the cytoplasm of living mast cells was determined by examining the cells in an interference microscope Leitz) when immersed in media of different refractiv indices The media consisted of series of concentrations of bovine serum albumin Armour fraction V) the tonicity of hich had been adjusted with sodium hloride to correspond to 0 9 per cent of this salt The mean olumes of 50 mast cells in 0 9 NaCl and in the highest protein concentration used proved to be identical (0 9 10^{-4} ml) showing that the tw solutions were isotone. For description of the technique, see Ross 19 1)

Statistical methods

The linear regressions of the ariables were calculated according to he method of least squares, and expressed in the form = bx + The *probability of b being equal zero was tested by Student* t-test When the number of observations as large enough, the cells were divided into classes with respect to fluorescence the gra densities of each lass being treated separately

Results

Concentration f H 5-HT and H 5-HTP by mast cells — After incubation of the cell suspensions for 10 min with 10 μc ml of H 5-HT or H 5-HTP most of the mast cells were labelled to some degree although the concentration of 5-HTP-radioactivity was onl around one sixth of that of 5-HT (Fig 2, 3 The cytoplasmic specific radioactivity could be increased by lengthening the incubation time an increase in the tim of exposure f the cells to the isotope solution by a factor 6 resulted in an increase in H HTP and H 5-HT concentration in the mast cells by factors 3.3 and 2.4 In all



Fig. 1 Optical path difference between cell and surrounding mounting medium over the diameter of mast cell, which had been fixed in formaldehyde vapours and mounted in glycerin, as recorded in scanning microinterferometer (Svensson 1957). The thickness of the dried cell was calculated for cellular density of 1.3 g/cm³. The horizontal lines A and B represent the thicknesses which absorb 90 and 98 per cent of the tritium β-rays, respectively (See text)

emer lucida, and the emission at 570 nm of individual mast cells, localized on the maps, was measured in fluorescence microspectrograph (Caspersson et al. 1965) as previously described (Ritzén 1967).

Autoradiography

When the fluorescence measurements had been completed, the specimens were transferred to dark room where strip film (Kodak AR 10) was applied on the cells according to the method originally described by Doniach and Pelc (1950). The autoradiographs were dried and stored in the dark at 4 C for 5—10 days, developed for 5 min in Kodak D19B, fixed and rinsed. Following this, the autoradiographs were mounted under coverslips in glycerin. The numbers of developed photographic grains were counted over mast cells that in the interference microscope were found to have mass thickness exceeding 0.05 mg/cm (For procedures, see Hale 1958, Zetterberg 1966). At this thickness, the tritium energy leaving the surface of the cell will be 90 per cent of that obtained from an object of infinite thickness (Maurer and Primbsch 1964). Consequently as long as the cell is thicker than this, the number of β-particles reaching the surface of the cell (and the photographic emulsion) will be function of the *concentration* and *not* of the *total content of radioactivity* in the cell. (For further discussions of self absorption of H β-rays, see Maurer and Primbsch 1964, Perry 1964, Zetterberg 1966). In the mast cells of the present investigation practically the whole cytoplasm showed thickness exceeding 0.05 mg/cm (Fig. 1). In few cases, where the layer of cytoplasm over the nucleus was so thin that it was revealed as lower grain density in the photographic film, the area and number of grains over the nucleus were subtracted from the respective figures for the whole cell. If not stated otherwise all the autoradiographic results are expressed as grains/μm², the area being determined from the diameters of the round cells.

In order to estimate the extent of diffusion of radioactively labelled compounds from the mast cells into the water during the application of the stripping film, cell suspensions containing the respective isotopes were brushed onto the sticky side of plastic tape, dried, and treated with formaldehyde gas as described above. The tape was cut into two pieces, one of which was dipped into water in the same way as it is done in the conventional strip film technique used in the present investigation. Then the tape was dried again, transferred to dark room and pressed onto a previously prepared slide carrying an AR 10 film with the emulsion side turned out from the glass. After exposure for 5 days, the tape was removed by xylene leaving the cells on the emulsion. The slides were then taken through series of alcohols to distilled water, developed, fixed and rinsed. The other half of the tape (including dried cells) was treated parallelly but without the initial immersion in water. This technique has been described by Hammarström et al. (1965) for autoradiography with water soluble isotopes. Following light staining in 0.1 per cent toluidine blue, the numbers of grains under 25 mast cells in each preparation were counted. The results showed average decrease in grain density under mast cells of 21 and 22 per cent for H-5-HT and H-5-HTP respectively following the immersion into water.

Autoradiographic reference system

In order to make possible conversion of the cellular concentrations of isotopes from relative (grains/μm²) into absolute (μc g) terms, reference system was included into the autoradiographic procedure. The technique which is described in detail elsewhere (Ritzén 1967b) was briefly the following. Sections of 4 to 6 μm thickness were cut from polymethyl-methacrylate block, which is uniformly labelled with H. These sections were placed on microscope slide, covered with emulsion and carried through the autoradiographic procedure parallelly with the specimen. The grain density over this section could be directly compared to the grain density over the infinitely thick cells (see above) and hence related to the specific activity of the methacrylate (in this case 57 μc/g).

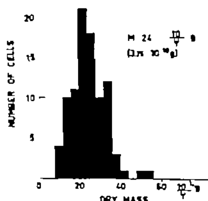


Fig 4 Histogram showing the frequency distribution of the dry mass of 89 rat peritoneal mast cells. The mean of the integrated optical path difference was transformed int bsolut weight by assuming hat γ equals 0.077 cm /g (see text)

much in different mammalian cells. Assuming that this figure is correct for mast cells, a mean dry mass of the mast cells of 3.25 × 10⁻ is obtained.

Knowing the absolute dry mass of the cell and the specific activity of the cytoplasm, the total amount of 5-HT taken up can be calculated. After incubati n for one hour this turned out to be around 10 per cent of the endogenous 5-HT and was therefore subtracted from the total amount of 5-HT in the respective cells, as measured by microfluorometry The fluorescence yield of 5-HT in this particular reaction was determined by parallel model experiments (see Ritzén 1967a) In the case of H 5-HTP this subtraction was not necessary since the uptake of this substance was only around one sixth of that of H 5-HT Furthermore 5-HTP gives only half the fluorescence yield of 5-HT (Ritzén 1967a)

The dry mass of the mast cell cannot readily be converted to volume of the living cell unless the concentrations of solids in the cell (w/v) in the living state is determined. The experiments with live mast cells mounted in bovine serum albumin of varying concentrations showed that the refractive index of the mast cell cytoplasm was matched at $n_D = 1.39$ corresponding to a 34 albumin solution (Ross 1961) where t had a minimum contrast in the interference microscope. This is considerably higher than other cell types. At this concentration, the cell nuclei and all the white blood cells showed a negative contrast The exact matching concentration of the latter cells was not determined, but seemed to be slightly above 22 per cent. Ninety per cent of the mast cells were matched in refractive index by albumin concentrations between 31 and 37 per cent. A few mast cells showed a cytoplasmic refractive index matching that of 40 albumin. These cells were often large and of an ovoid shape.

According to Lagunoff t al (1964) the mast cell granules consist of 30 per cent heparin, the rest of the dry mass being mainly made up of protein. Since a 15 aqueous solution of heparin (Vitrum, Stockholm) showed a refractive index (1.348) that was not very far from that of protein at the same concentration (1.362) the specific refractive increment of mast cell granules in water can be assumed to be the sam as that of protein (0 18 cm g see Hale 1958) Thus the cytoplasmic refractive index can be directly transformed into concentration (solid Ross 1961)

TABLE I A mmary of th correlations between endogenous 5-HT content, dry mass nd concentration of the isotope in individual mast cells.

	F t DM	F/DM to DM	AR t DM	AR to F	DM × AR to F	AR to F/DM
H 5-HTP						
corr oeff. ( )	0 482	—0.512	—0.3.2	—0.141	0.451	0.303
regr coeff. (b)	0.233	—0 010	—0.001	—0 001	2.53	0 183
p that b = 0	< 0.01	< 0.001	< 0.05	< 0.2	< 0.02	< 0.1
n	31	31	31	107	31	31
H 5-HT						
corr oeff. ( )	0 393	—0.379	—0.081	—0.321	0.245	0.158
regr coeff. (b)	0.381	—0.019	—0.003	—0 010	0.53	0.193
p that b = 0	< 0 02	< 0.03	< 0.7	0.01	< 0 2	0 4
n	33	33	33	93	33	33
H-DOPA						
corr coeff. ( )				0.014		
regr coeff. (b)				0 000		
p that b = 0				0 9		
				81		

DM = dry mass
F = fluorescence (dica ing total content of endogenous 5-HT)
AR = gra density (indicating concentration of isotope in the dried cell)
F/DM = rel ti cellula concentration of endogenous 5-HT
DMxAR = total amou t of isotopes taken up (arbitrary units
= correlation coefficient
b = regression coefficient
p = probability
n = umber of cells measured

The results of the calculations of correlations between the various parameters measured are summarized in Table I and will only be briefly described below

Concentrations and total amounts f endogenous 5-HT in cells of different sizes — The relative concentrations of 5-HT in the mast cells was calculated as the quotient fluorescence dry mass, the tv o parameters representing total amount of 5-HT and cell mass (volume) respecti ely There proved to be a highly significant negative correlation between concentration and dry mass, larger cell generally having a lower oncentration of 5-HT than smaller ones. In spite of this, the total content of endogen us 5-HT was higher in the large cells than in the small, the larger mass of the former over compensating the decreasing concentration (Table I and Fig 5)

Relation of concentrating capacity of mast cells to dry mass — There was a clearcut negative correlation between these two variables as far as H 5-HTP was concerned, the gram density decreasing with increasing dry mass. For H 5-HT however this decrease was not significant. The variations between the individual cells were, as in the other plots, profound (Table I and Fig 6)

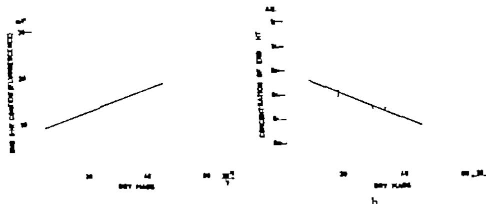


Fig. 5 a and b. Diagrams showing the correlations between total content (5a) or concentration (5b) of 5-HT and dry mass of the same mast cell. The relative 5-HT content was determined by microfluorometric and the dry mass by microinterferometric methods. Each point represents one cell.

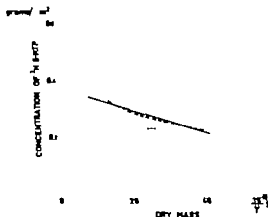


Fig. 6. The solid line represents the calculated linear regression of the cellular concentration of radioactivity to dry mass of mast cells after incubation with ³H-5-HTP for one hour. The dashed line illustrates the variation of the ratio surface to volume of a sphere with increasing volume.

Relation of isotope concentrating capacity to endogenous 5-HT. — When the grain density was plotted against the fluorescence of the same cells, it became evident that for the individual cell, the concentrating capacity could not be predicted from its total fluorescence. However on the whole there proved to be a highly significant *negative* correlation between grain density and fluorescence what concerns ³H 5-HT. This correlation is more pronounced when the population is divided into classes, the means of which are depicted in fig. 7 in addition to the individual cells. For ³H 5-HTP the same tendency was found, but not for ³H DOPA, the uptake of which showed no correlation whatsoever with the fluorescence. (See Table I and Fig. 7.)

Total uptake of isotopes in relation to 5-HT content. — A relative figure for the total content of radioactivity in each cell was obtained from the product of grain density (concentration of isotope) and dry mass. In the case of 5-HTP it was found that an increase in fluorescence was accompanied by an increase in total ³H 5-HTP uptake,

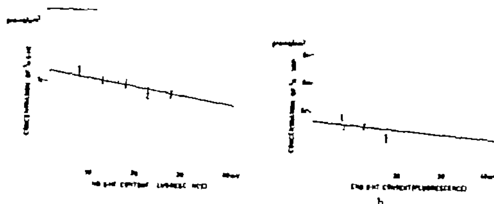


Fig. 7 a and b. The concentration of radioactivity in mast cells after incubation for one hour with H-5-HT (7) and H 5-HTP (7b) plotted galnst the content of endogenous 5-HT in the same cell, as measured by microfluorometry Filled circles with vertical bars represent means nd standard errors of the specific *radioactivity of cells falling within class width of 5 fluorescence units (mV).*

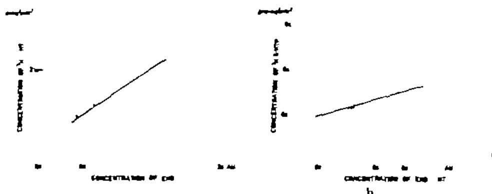


Fig 8 nd b. Diagrams illustrating the correlation between the concentrating capacity of mast cells owards exogenous H 5-HTP (8a) or H 5-HT (8b) and the endogenous concentration of the amine the same cell.

although the variations among the individual cells were pronounced. The same tendency was found for H 5-HT (Table I) these cells showing a still more accentuat ed individual variation (Fig 8)

Discussion

The tritiated compounds used in the present investigation were randomly labelled, and hence metabolic products as well as the original compounds were labelled and displayed as *photographic grains in the autoradiograms. For 5-HT this does not seem* to be any serious complication Furano and Green (1964) did not find any metabolic products of 5-HT in peritoneal mast cells after administration of ¹⁴C-5-HT The situation is somewhat different for 5-HTP being the immediate precursor of 5-HT Observations on the rate of synthesis of 5-HT by normal mast cells incubated with moderate concentrations of 5-HTP seem to be lacking Thus, it is not known to what extent the observed radioactivity in the peritoneal mast cells, seen after incubation

with H 5-HTP for one hour represents newly formed H 5-HT Nevertheless, the concentration of radioactivity indicates the uptake of the amino acid, even if part of it has been decarboxylated in the cell.

The dry mass of the mast cell is to a major part determined by the cytoplasmic dry mass, since the nucleus contributes with only around one per cent. (Ottoson *et al.* 1958.) The main part of the cytoplasmic dry mass is composed of the very dense amine storage granules, which almost fill the cytoplasm. Therefore, the dry mass of a mast cell will to a large extent be a measure of the amount of amine-storing structures. The content of 5-HT in a normal mast cell was found to be less than one per cent of the total dry mass, and hence variations in 5-HT content would not be detected by the interferometric methods.

In no case was there any uptake of radioactivity by the white blood cells in the cell suspension. With the concentration of radioactivity used, a free passage of isotopes into the cell, equilibrating the concentration of radioactivity on both sides of the cell membrane, would have resulted in a grain count of 0 05 grains /μm If this figure was subtracted from the total grain density after 10 and 60 min of incubation with H 5-HTP the active uptake mechanism had contributed to the concentration of intracellular radioactivity with 0 05 and 0.28 grains/μm respectively indicating a fairly constant rate of uptake over this period of time The corresponding figures for H 5-HT however were 0.55 and 1 40 grains/μm thus the rate of uptake of the amine was higher during the initial period than after one hour (Fig 3)

There seems to be two possible major factors that determine the uptake of amino acids and amines into mast cells one is an active pump" mechanism at the cell membrane, the other is the capacity to store the ubstances taken up. If the "membrane pump was the limiting factor fo uptake, and no re-exit occurred the total amount of intracellular radioactivity would ha e been proportional to the total area of the cell membrane, provided that this is the location of the "pump" and that the uptake is uniform over the whole membrane If, on the other hand, the storage capacity is the facto that limits uptake the latter will be independent of the total membrane area.

In order to investigat which of these two mechanisms that was probable for the uptake of H 5-HTP the variation of the quotient surface/volume of a sphere with increasing volume was depicted in the plot representing grain density (concentra tion of H) versus dry mass (volum) of the cells. It was found to f llow the calculated linear regression line of the cells very closely (Fig 6) This seems to indicate that for the uptake of the precursor amino acid the first of the two possibilities is of major importance. On the other hand, for the uptake of H 5-HT during a period of one hour some other factor seems to be rate-limiting After incubation with this substance the concentration of H in the mast cells was practically independent of their dry mass (Tabl I) However the total amount of H 5-HT taken up expressed by the product of dry mass and grain density generally showed an increase w th increasing size of the cell (Tabl I)

The ptake f 5-HT into normal mast cell has also been studied by Furano

Green (1964) These authors also noted that mast cells had an extremely efficient uptake mechanism for 5-HT being capable of concentrating exogenous ^{14}C-5-HT 78 times compared to the concentration in the surrounding medium. This figure is around twice as high as that obtained in the present investigation, when the H-5-HT in the mast cells is expressed in terms of concentration per unit wet volume instead of unit dry mass of the mast cell. This difference might be due to several circumstances, including the fact that Furano and Green (1964) made their investigations on mast cells that had been isolated by density gradient centrifugations, and that these authors used another kind of incubation medium. Also it has been noted (Moran *et al* 1962) that the content of amines in rat mast cells might vary considerably on different occasions, even within the same strain. However it cannot be excluded that part of the exogenous H 5HT newly taken up was so weakly bound in the mast cells that it was released during the preparation of the microscopic specimens.

In most cell populations, large cells are believed to be of older age than small ones. If this is true for rat peritoneal mast cells older cells seem to have declined in activity Generally small cells showed higher concentrations both of endogenous (5-HT) and exogenous (^{3}H-5-HT and H 5-HTP) substances, after incubation with the latter than did larger cells. This is in contrast to the enterochromaffin cells in the mouse gastric mucosa, where an increasing age was accompanied by an increase in concentration of 5-HT but a decrease in 5-HTP uptake activity (Hammarström *et al.* 1966)

The results concerning uptake of ^{3}H DOPA into mast cells are of a preliminary nature however they confirm earlier observations that rat mast cells (Hempel 1963 Enerbäck 1966) are able to concentrate this amino acid, as are mouse mesenteric mast cells (Eränkö and Kauko 1965) The results cannot be quantitatively compared to those of 5-HT and 5-HTP uptake, since the degree of diffusion of H DOPA from the cells during the autoradiographic process was not determined. There was, however a remarkably large variation within the group of cells with respect to concentration of the isotope in the cytoplasm.

The main conclusion of the present investigation is that the rat peritoneal mast cells show a very marked intercellular variability some cells having a 5-HT content and uptake capacity that is 4 to 5 times higher than the median value. The finding that large cells have a lower concentration of endogenous 5-HT than the small ones seems to be a reflection of a lower metabolic activity of the large cells. This in turn might be due to unfavourable proportions between cell surface and cell volume, the former being the rate-limiting factor for uptake of precursor amino acid. The extremely efficient uptake mechanism for 5-HT that was displayed by the rat mast cells provides these cells with a possible function not only as a 5-HT synthesizing and secreting structure but also as a system for neutralizing tissue 5-HT by incorporation into the mast cells.

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Electrical and Mechanical Activity of Human Respiratory Muscles during Voluntary Inspiration

By

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Abstract

Viljanen, A. A. *Electrical and mechanical activity of human respiratory muscles during voluntary inspiration.* Acta physiol. scand. 1967. 70. 54—56.

It has been shown, that in a certain range of inspiration there exists a linear relationship between the total number of electromyogram impulses during inspiration and the first time integral of the muscular inspiratory work of breathing. During one inspiration the number of electromyogram impulses is increasing in a linear relationship to the pressure impulse.

Earlier experiments show that there is a close relationship between the mechanical respiratory functions and the electric activity in human intercostal muscles. A linear relationship was demonstrated between the electromyogram impulse frequency at the end of inspiration and the muscular inspiratory work of breathing. The electromyogram impulse frequency determined four seconds after the end of inspiration and the subject holding his breath with the glottis open, was in a linear relationship to the alveolar relaxation pressure (Viljanen *et al.* 1964).

In further experiments using the same methods as earlier the electrical activity of human intercostal muscles has been examined during inspiration. The electromyograms were taken using both surface and concentric needle electrodes. 15 healthy subjects were examined and 50—90 inspirations of each person have been recorded.

It was shown that in a certain range of inspiration there is a linear relationship between the total number (n) of electromyogram impulses discharged during the inspiration and the first time integral (H_t) of the muscular inspiratory work of breathing, so called "physical action" (dimension cm H_2O × sec × 1 [illegible], compare Bergström 1962) (Fig. 1). During one and the same inspiration however the number of electromyogram impulses did not increase in a linear manner with the corresponding physical action (Fig. 2). Instead there was observed a linearity between the number of electromyogram impulses and the pressure impulse ($I = \int p\,dt$) where pressure (p) is equal to the sum of the dynamic and alveolar relaxation pressure

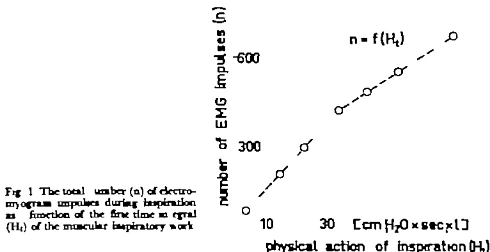


Fig 1 The total umber (n) of electromyogram impulses during inspiration as function of the first time integral (H_t) of the muscular inspiratory work

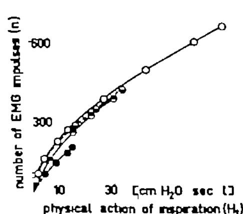


Fig. 2.

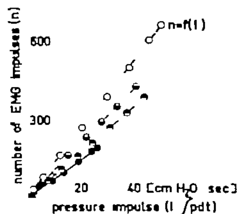


Fig 3

Fig 2 The number (of electromyogram impulses during 4 inspirations of differen deepness, as function of the first time integral H_t of the muscular inspiratory work.

Fig 3 The number) of electromyogram impulses during 4 inspirations of differen deepness, as function of pressure impulse (I)

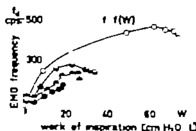


Fig 4 The dynamic electromyogram impulse frequency f_d during 4 inspirations of different deepness, as function of the muscular inspiratory work (W)

Acta physiol. scand. 1967 70. 57—68

From the University Institute for experimental Medicine, Division of Endocrinology Copenhagen, Denmark

Insulin Binding Proteins in Normal Serum Qualitative and Quantitative Studies in vivo in Cats

By

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Received 18 November 1966

Abstract

GJEDDE, F *Insulin binding proteins in normal serum Qualitative and quantitative studies in vivo in cats* Acta physiol. scand. 1967 *70* 57—68.

After 1 infusion of homologous ^{131}I labelled insulin to cats, the serum radioactivity decreased exponentially and in logarithmic plot three different slopes were observed. The portion with the steepest slope had half-life of from 4 to 8 min and corresponded to the elimination of free insulin.

The intermediate portion of the curve was caused by the association of bou 18 per cent of the injected radioactivity with an alpha-one globulin. The half-life of this complex was 150 min being caused by equilibration of the labelled substance between intra-and extravascular space. Of this radioactivity associated with the alpha globulin 30 per cent could be precipitated using double immunoprecipitation for insulin. By electrophoresis on starch block and starch gel the radioactive globulin was characterised as fast alpha-one globulin. By gel filtration on Sephadex G 200 it was eluted with the albumin peak. Only 15 per cent of the radioactivity associated with the alpha-one globulin could be precipitated by 80 per cent saturation with ammonium sulphate, whereas 100 per cent could be precipitated with trichloroacetic acid.

When the radioactive alpha-globulin was infused into four other cats, it was diluted with volume corresponding to 4.7 per cent of the body weight after 24 hrs the space had expanded to alue corresponding to 19 per cent of the body weight. After equilibration, fall of the protein-bound radioactivity of serum and an excretion of radioactivity in the urine corresponding to half life of 50 and 52 hrs was observed.

No association of radioactivity with the macroglobulins could be demonstrated in vivo.

Preliminary investigations showed that the insulin-like activity (ILA) of serum, measured by the adipose tissue assay was increased from 100 to more than 100 000 microunits per ml serum after dialysis against tap water at room temperature The substance producing the increment was localized in a precipitate of denatured globulins (Gjedde 1960)

Since the increased acti ty was partly inhibited by antiinsulin serum, and since the activity in cats showed a decay after pancreatectomy corresponding to a half life of 3 days the hypothesis was proposed that part of the circulating endogenous insulin is associated with certain euglobulins, thereby altering tu volume of distribution and its rat f catabolism (Gjedde 1964)

(Fig. 3). It was noticed that the coefficient of the relationship is determined by the deepness and the velocity of the inspiration.

Although the electromyogram frequency (f_e) at the end of inspiration is in linear relationship to the inspiratory muscular work (W), this is not the case in the different phases of the same increasing inspiration (Fig. 4). During one inspiration the electromyogram impulse frequency and the average power ($\bar{P} = W/t$) of the inspiratory muscles seem to be over a wide range in a linear relationship.

The results show also that the same relationship between the electrical and mechanical activity as found in the intercostal muscles also exists in the muscles of the diaphragm.

Acknowledgement

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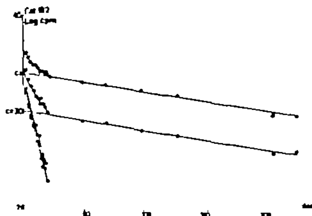


Fig. 1 (Cat B 2): Elimination of trichloroacetic acid (TCA) precipitable radioactivity (○) and radioactivity precipitated with double immunoprecipitation for insulin (Im.) (●) after intravenous infusion of ^{125}I labelled cat insulin into cat. The curves of radioactivity were graphically resolved int two separat exponential func tions of time b3-TCA (T/2 6 min.) ○ – – – ○ b3-Im. (T/2 6 min.) ● – – – ● b2 TCA (T/2 155 min.) ○ – – – ○ b2 Im. (T/2 155 min.) ● – – – ●

pH 7 4 I was injected intravenously into 4 cats under pentobarbital anesthesia Blood samples were drawn from th external carotid artery t hosen time intervals for 2 hrs, whereupon the cats were placed in metabolic cages, permitting quantita urine collections. T prevent incorpora tion of radioacti iodine int the thyroid hormones, sodium iodide was added to the milk at final concentration of 0 01 per cent T obtain large diuresis the cats were kept on milk exclusively nd the urine as collected daily for 9 days.

Measurement of protein-bound radioactivity

100 µl of serum was precipita ed with 1 ml of 5 per cent trichloroacetic acid (TCA) or by double immunoprecipitation of insulin, and the radioactivity was measured with Frieseke Hoepfner well-type scintillation counter (Gjedde 1966)

Analysis of data

The radioactivity of serum precipitates was plotted on logarithmic scale (ordinate) against time on linear scale. According the method of Matthews (1957) the composite part of the curv for the protein-bound radioactivity was graphically resol ed int separate lines, by subtraction of the extrapolated straight portion of the curv from the measured amount of radioactivity and plotting the differenc as separate function of time

I th short term experiments, series A, B and C, the curv for the radioactivity was resolved into one rapidly decli ng line (slope b 3 and one of intermediate slope (b 2) (Fig 1) I the long erm experiments, the radioactivity curve was resolved int one intermediately (b 2) and one slowly (b 1 declining line (Fig 4)

The intercept with he ordina ( ) of the three lines of different slopes (b) were determined, c1 being he intercept of the most horizontal exponential line (with slope b1) c2 the intercept of he intermediate exponential line (slope b2) The slopes were obtained from the formula

$$b = \frac{0.693}{T/2}$$

The lue for *1* (see Fig 4) as obtained by extrapolation of he curv with the slope b 1 It denotes the serum concentration of he radioactivity bound to alpha-one globulin, if equilibrium between the intra och extravascular distribution spaces had been reached instantaneously before ny degradation had occurred The corresponding exponential line, with the slope *b 1* reflects he rate of degradation of the labelled alpha-one globulin

The alue for *2* represents the intra ascular concentration of he radioactivity associa ed with the alpha-one globulin I short term experiments, this alue is obtained by extrapolation to zero time see Fig 1 I he long term experiments, where labelled alpha-one globulin was injected intra enously this alue was measured directly The corresponding decay curv with the slope *b 2* depicts the rat which equilibration between the intra and the extravascular distribution olumes is obtained

The steep exponen ial line ith the slope *b 3*, expresses the ra which free iodinated insulin is eliminated from serum nd was obtained by subtracting he extrapolated values of *b 2* from the measured lues of protein-bound radioactivity in short term experiments The curve is drawn ith dotted line in Fig 1 The corresponding value for 3, an expression of the concentra ion of

free insulin at zero time was not used in the calculations on account of the relatively long period of infusion of insulin, in regard to the short half life of the latter

The *degradation rate* was expressed as the daily excreted urinary radioactivity in per cent of total retained radioactivity being the difference between the injected and the accumulated excreted urinary radioactivity

The volume of distribution was calculated by dividing the injected dose of radioactivity with the radioactivity of 1 ml serum, measured at or extrapolated to the start of the experiment.

Results

1 *Short term experiments A and B series*

As seen from Fig 1 the protein-bound (TCA precipitated) radioactivity becomes a single exponential function with time after approximately 30 min. By extrapolation and subtraction, the curve is resolved graphically into two separate exponential functions of time, yielding a steep line (b 3) with a half life of 6 min, and a flat curve (b 2) with a half life of about 155 min. As shown by Fig 1 this type of analysis gives a reasonable good fit with the data.

2 min after the end of the infusion, the extrapolated value for the slow curve constitutes more than 50 per cent of the total protein bound (TCA-precipitable) radioactivity (*Table II*)

From the figure and from the table it is also apparent that the curve of the immunologically precipitable radioactivity is roughly analogous to the curve of total TCA precipitable radioactivity although the former curve is depressed in relation to the latter constituting from 66 to 36 per cent of the latter (Table II) The apparent half lives of the two components of the two curves are very similar being 7 and 8 min for the steep component and 136 and 151 min for the flat curve (Table I)

By using the extrapolated value of c 2 for the slowly eliminated component of protein-bound radioactivity and assuming a volume of distribution of 4 7 per cent

TABLE I *Rate of elimination of trichloroacetic acid precipitable radioactivity and of radioactivity obtained by immunoprecipitation for insulin A and B series*
Mean ± 1 S D

	TCA precip	Immunol. precip
Half-life of b 3	8 ± 1.5 min (N:6)	7 ± 1 4 min (N:6)
Half-life of b 2	155 ± 21 min (N 6)	136 ± 19 min (N 6)

TABLE II The relation between radioactivity of serum precipitated by immunoprecipitation of insulin and by trichloroacetic acid, at 2 and 60 min after the injection of labelled insulin, and between the corresponding values for c 2 extrapolated to 2 min A and B series. Mean ± 1 S.D

Immunol. precip radioactivity × 100 / TCA precip. radioactivity		Immunol c 2 × 100 / TCA c 2	TCA c 2 100 / Total TCA
2 min	60 min		
66 ± 9 % (N 6)	36 ± 9 (N 6)	35 ± 9 % (N:6)	56 ± 3 (N 6)

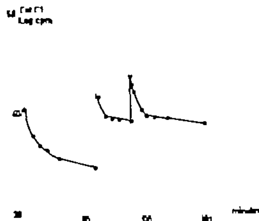


Fig 2 (Cat C 1) Radioactivity of serum, precipitated with trichloroacetic acid after 3 i. injections of ^{131}I labelled cat insulin with time intervals of 60 min. 1 connection with the third injection of labelled insulin fall of the blood pressure from 100 to 70 mm Hg was observed.

of the body weight (see under long term experiments) and relating this to the amount of injected radioactivity the degree of incorporation can be calculated. This was found to be 17.9 ± 4 9 per cent of the injected dose of labelled insulin (Mean ± 1 S.D., N 6) corresponding to about 200 microunits per ml serum.

2 min after the end of the infusion of insulin the apparent volume of distribution corresponded to 15 ± 5 6 per cent of the body weight (Mean ± 1 S.D., N 6)

2. *Short term experiments — C series*

The experiments with the animals C 1 and C 2 differed from the A and B series in that repeated infusions of shorter durations were employed. The apparent volume of distribution was 12 ± 2 per cent of the body weight 30 sec after the insulin was injected, rising to 18 ± 3 per cent 90 sec later The accumulation of radioactivity in the slowly eliminated fraction seemed to be additive. Only 10 4 ± 4.3 per cent (Mean ± 1 S.D N 5) of the infused dose was incorporated (Fig 2) Similarly the values of b 3 and of b 2 were smaller than those of the A and B series, as the half lives were 4 ± 0.25 and 102 ± 28 minutes respectively (Mean ± 1 S.D N 5)

In the experiments C 3 where a cat was given a constant infusion of 925 microunits labelled insulin per kg per minute for 120 min, a rise of protein-bound radioactivity of serum was observed (Fig 3) The increment declined with time from a value corresponding to 57 microunits insulin per ml serum for the first 30 min. to a value corresponding to 28 microunits per ml in the experimental period for 90 to 120 min, or to 9 5 and 4 7 per cent of the injected dose.

These findings indicate that a saturation with radioactivity of the slowly eliminated component was not obtained. Nor would it seem that the continuous infusion of labelled insulin had been exceeded by the degradation capacity

3 *Characterization of the protein-bound radioactivity*

To characterize the slowly eliminated radioactive protein fraction separation method for proteins were used. When serum, taken more than two hours after the infusion

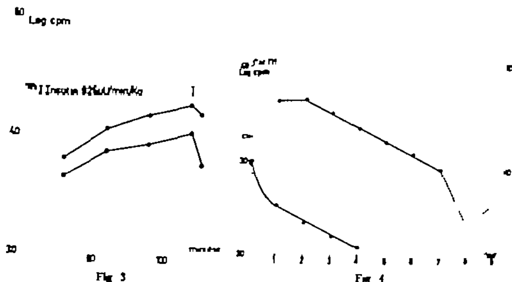


Fig 3 (Cat C 3) Radioactivity of serum, precipitated with trichloroacetic acid (●) and by double immunoprecipitation of insuli ( ) of cat hat received constant infusion of ¹³¹I labelled cat insulin for 120 min

Fig 4 Radioactivity of serum, precipitated with trichloroacetic acid (●) nd total radioactivity excreted per 24 hrs ( ) in the urine of ca that had received an infusion of radioactiv lpha-one globulin. Th slope b2 — inact — corresponds to the equilibration of the protein-bound radioac tivity between intra nd extravascular olume of distribution, and the slope b1 to the metabolic degradation of the labelled compound. The half-lives corresponding to b2 is 150 min t b1 (serum) 48 hours and t b1 (urine) 51 hrs.

of insulin, was dialyzed against phosphate-saline buffer at pH 7 4 the amount of radioactivity inside the membrane showed an initial fall due to removal of degradation products and then remained constant and was fully precipitable with trichloroacetic acid.

Preparative electrophoresis on starch block of 5 to 10 ml of serum, taken 180 min after the injection of insulin, showed that all radioactivity migrated as an alpha-one globulin. By immunoprecipitation of nsulin, 30 per cent of this radioactivity could be precipitated whereas 90 per cent of the ¹³¹I labelled insulin, added to serum immediately prior to electrophoresis, could be recovered by immunoprecipitation.

In control experiments in order to correct for coprecipitation it was found that less than two per cent of the radioactivity precipitated, when normal guinea pig serum was used instead of serum from guinea pigs immunized with insulin.

Horizontal starch gel electrophoresis revealed that a peak of more than 80 per cent of the radioactivity was localized in a faintly coloured protein band mmediately behind, but clearly separated from the albumin in contrast, starch gel electrophoresis of serum, to which an equal amount of radioactive insulin was added just prior to electrophoresis, showed that about 90 per cent of the radioactivity migrated in front of the pre-albumin.

Gelfiltration on Sephadex G 100 or G 200 gave a symmetrical radioacti ty curve w th a maximum 1 to 2 fractions after the main protein peak (Fig 5) while 95 per

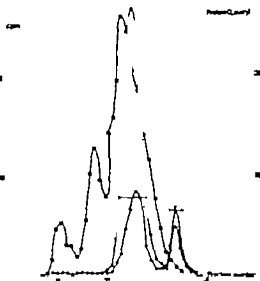


Fig. 5. Gel filtration on Sephadex G 200 of serum from cat, which had received constant infusion over 15 min. of ¹²⁵I labelled cat insulin. The serum was obtained 35 min after the end of the insulin infusion, and dialyzed against phosphate buffer 10 mM, pH 7.4, containing 0.9 per cent NaCl. The same buffer was used for the gel filtration. Protein (Lowry units) (■) The major part of the protein-bound radioactivity (○) was eluted immediately after the main protein peak. 30 per cent of the radioactivity of this peak could be precipitated by double immunoprecipitation of insulin (●) The free insulin was eluted last. 90 per cent of this fraction were precipitated by immunoprecipitation. I 1—I bound radioactivity 2 free insulin.

cent of the radioactivity of labelled insulin, added to serum less than 10 minutes before gel filtration, was eluted after the protein.

Repeated immunoelectrophoretic investigations, using a potent anti-serum against cat serum and cat serum with a high concentration of radioactivity associated with the alpha-one globulin, failed to produce any radioactive precipitation arcs by autoradiography

When ammonium sulphate (pH 8.0) was added to 80 per cent saturation to dialyzed serum that was taken from cats more than 120 minutes after the termination of the insulin infusion, it precipitated about 15 per cent of the radioactivity In contradistinction to this unusual solubility more than 80 per cent of the radioactivity was precipitated when labelled insulin was added to serum immediately before addition of ammonium sulphate to 80 per cent saturation.

It is concluded from these results that between 10 and 20 per cent of the radioactivity of infused labelled cat insulin is bound *in vivo* to protein, fully precipitable with trichloroacetic acid, but soluble in high concentration of ammonium sulphate, with an electrophoretic mobility in starch block and in starch gel electrophoresis corresponding to an alpha-one globulin and with a molecular weight several times that of labelled crystalline insulin, as deduced from the behavior on Sephadex gels. When the properties of this labelled protein are compared with the properties of the labelled alpha-one globulin found after *in vitro* incubation of serum with labelled insulin (Gjedde 1966) an identity of these two proteins seems to be indicated.

However in contrast to the *in vitro* experiments, radioactivity from labelled insulin was never observed to associate with the macroglobulins in these *in vivo* experiments.

In other experiments of a preliminary character ¹³¹I labelled insulin was injected to cats, either into one of the portal tributaries by constant infusion over several



Fig 3.

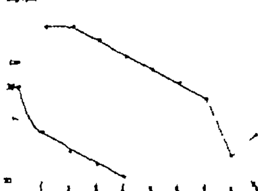


Fig 4

Fig 3. (Cat C 3) Radioactivity of serum, precipitated with trichloroacetic acid (●) and by double immunoprecipitation of insulin ( ) of cat that received constant infusion of ¹³¹I labelled cat insulin for 120 min.

Fig 4 Radioactivity of serum, precipitated with trichloroacetic acid (●) and total radioactivity excreted per 24 hrs ( ) in the urine of a cat that had received an infusion of radioactive alpha-one globulin. The slope b2 — inset — corresponds to the equilibration of the protein-bound radioactivity between intra- and extravascular volume of distribution, and the slope b1 to the metabolic degradation of the labelled compound. The half-lives corresponding b2 a 130 min to b1 (serum) 48 hours and to b1 (urine) 51 hrs.

of insulin, was dialyzed against phosphate-saline buffer at pH 7 4 the amount of radioactivity inside the membrane showed an initial fall due to removal of degradation products and then remained constant and was fully precipitable with trichloroacetic acid

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Gelfiltration on Sephadex G 100 or G 200 gave a symmetrical radioactivity curve with a maximum 1 to 2 fractions after the main protein peak (Fig. 5) while 95 per

TABLE IV. Daily urinary excretion of radioactivity after intravenous infusion of labelled alpha one globulin, expressed as per cent of retained radioactivity (obtained by subtracting cumulative urinary excretion from injected radioactivity)

Period	Excreted urinary radioactivity in per cent of retained				
	Cat D1	Cat D2	Cat D3	Cat D4	Mean ± 1 S.D
1.day	19 8 p.c.	17.6 p.c.	21.8 p.c.	12.7 p.c	15.5 ± 4.9 p.c.
2.day	25.2 p.c.	24.3 p c.	18.7 p.c.	25.7 p.c	23.5 ± 3.2 p.c.
3 day	24 7 p.c.	22.9 p.c	20.7 p.c.	17.0 p c.	21.3 ± 3.3 p.c
4.day	23 4 p.c.	23 4 p.c.	18 5 p c.	16.8 p	21 0 ± 3.0 p.c
5 day	21 1 p.c.	24 9 p.c.	22 6 p.c.	14.5 p.c	20.8 ± 4.5 p.c
6.day	24 0 p	19.3 p c.	23.5 p.c.	12.5 p	19.8 ± 5.3 p
7.day	21.5 p c.	15.6 p.c	24.9 p c.	13 4 p.c.	18.8 ± 5.3 p.c
8.day	6 0 p.c.	22.9 p	24 0 p	5.8 p.c	14 7 ± 10 1 p.c.
9 day	11.3 p.c	15 7 p.c.	26.3 p	10.2 p.c.	15.9 ± 7.3 p.c.
Mean ± 1 S D	19.6 ± 6 6	20 5 ± 3.8	22.3 ± 2.6	14.3 ± 2.6	18.7 p c.
	Mean 19 1 p c.				

The urinary excretion of radioactivity reflecting the metabolic degradation rate, gave a straight line of good fit when the logarithm of radioactivity was plotted against time (Fig 4) The half-life determined by this curve was in good accordance with the slow decline of serum radioactivity with an average value of 52 hours. No excess radioactivity was excreted in the first period of the experiment, indicating metabolic uniformity and absence of major denaturation of the radioactive alpha-one globulin. This is evident since the relation

Radioactivity retained

Daily urinary excretion of radioactivity × 100 was constant at 19 per cent for the first 7 days after the infusion (Table IV)

1 *Elimination rate of labelled insulin from blood*

Inconsistency in the values for the disappearance rate of insulin in blood exists in the literature.

In rabbits, Scott et al. 1958 and Berson, Yalow and Volk 1957 have reported average plasma disappearance rates with half-lives about 25 minutes for the rapidly eliminated component. In man, Berson *et al* 1956 found an initial disappearance rate with a half-life of about 40 min, whereas the figures of Welsh *et al.* 1956 indicate an appreciable shorter half-life. Similarly Bollinger and Stephens 1964 found an apparent half life of 15 min for the rapid component of the disappearance curve in man Using [35]S (sulphatized) and [131]I labelled insulin, Haugaard *et al.* 1954 found a fast initial elimination of the radioactivity of whole blood in dogs and rats in contrast, Ricketts, Wildberger and Regut 1963 calculated the half-life of labelled insulin in rats to be 17 min at an average.

hours, or subcutaneously The results showed that no radioactivity could be demonstrated in the macroglobulins by starch gel electrophoresis. Nor was there any association of radioactivity with the macroglobulins after gel filtration or by starch gel electrophoresis, when labelled alpha-one globulin had been infused into cats.

4 *Evaluation of the metabolic degradation rate of the radioactivity associated with alpha-one globulin. Series D*

After the infusion of labelled insulin in the short term experiments a fraction, increasing with time, of the radioactivity of serum could not be precipitated by trichloroacetic acid, although 100 per cent of the radioactive insulin was precipitable after addition prior to injection. 30 minutes after the infusion of labelled insulin the percentage of non-TCA precipitable radioactivity varied from 20 to 30 per cent of the total serum radioactivity This figure indicates that the steep phase of the disappearance curve (b 3) of protein bound serum radioactivity is caused by the destruction of free insulin to at least a major part.

In order to determine the metabolic degradation rate of the slow component of the disappearance curve for serum radioactivity experiments were performed with passive transfer of alpha-one globulin-bound radioactivity The infused radioactive alpha-one globulin was found to be diluted immediately after the injection with a volume corresponding to 4 7 ± 0.25 per cent of the body weight after 24 hours the volume had expanded to 18.6 ± 3.5 per cent of the body weight (Mean ± 1 S.D., N 4)

Th initial disappearance of radioactivity from serum had an average half-life of 134 min (Table III) which was in good agreement with the figures for the apparent half-lives of the slowly eliminated component of the A and B series. This decline was accompanied by an appearance of less than one per cent of the radioactivity in trichloroacetic acid soluble form. After the initial equilibration, a slow decline of protein-bound radioactivity of serum was observed that corresponded to a half-life of 50 hrs.

TABLE III The rate of elimination of radioactivity of serum af er th infusion of labelled alpha-one globulin into cats. The radioactivity precipitated with trichloroacetic acid is expressed as per cent of the value 2 minutes after the end of the infusion. D series

Time after end of infusion	Mean ± 1 S D	Number of cats
62 min	65 0 ± 6.0 p.c.	4
122 min	57 0 ± 6 0 p.c.	4
24 hrs	18.6 ± 3.5 p.c.	4
48 hrs	13 9 ± 2 3 p.c.	4
72 hrs	9 2 ± 1.8 p.c.	4
96 hrs	6.5 ± 1.5 p.c.	4
120 hrs	5.0 ± 1.0 p.c.	2
144 hrs	3.8 ± 1.0 p.c.	2
168 hrs	2 3 ± 0.1 p.c.	2

difference was found to be attached to a protein migrating as an alpha globulin. Although control experiments prior to injection showed that less than two per cent of the labelled insulin migrated in paper electrophoresis, Berson Yalow and Volk 1957 nevertheless concluded that the radioactive alpha globulin which they calculated represented more than 10 per cent of the injected dose was caused by "preparation damage". This radioactive serum protein was eliminated slowly from the blood.

From studies of labelled serum proteins it is well known that the preparation with the longest biological half life is the least denatured one (MacFarlane 1957). By analogy it should be expected that the rapidly eliminated component of the composite curve of radioactivity of plasma after injection of labelled insulin is caused by denatured protein, and that the slow component represented native insulin.

In accordance with this, it has been shown that ensured denaturation, such as inactivation by alkali results in an increased rate of elimination of radioactive insulin (Lee 1957). The only observation in favour of the view that the slowly eliminated alpha globulin is caused by denatured radioactive insulin is given by Berson and Yalow 1957 who found that ^{131}I labelled insulin, that had been heavily irradiated with roentgen rays, was eliminated slowly *in vivo* after this treatment. However they also found that the radioactivity in paper electrophoresis migrated with *all* the serum proteins. In spite of this important difference in electrophoretic mobility between the irradiated insulin and *in vivo* labelled alpha globulin, Berson and Yalow 1961 maintained that the labelling of alpha globulin after injection of radioactive insulin was caused by an association of denatured insulin with a normal plasma protein.

Whether the radioactive alpha globulin represents a binding of native or of denatured insulin to a serum protein will be discussed in details in connection with investigations of the insulin like activity of serum protein fractions (Gjedde, in preparation).

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Insulin Binding Proteins in Normal Serum. The in vitro Association of Homologous ¹³¹I Labelled Insulin and Normal Serum Proteins

By

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Abstract

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By incubation in vitro of normal human serum with ¹³¹I labelled insulin, association of radioactivity with serum protein fractions was demonstrated. Previous findings of an association with two macroglobulins were confirmed and an association with a third fraction observed. The macroglobulins were identified as alpha-2 M and beta-2 M globulin by starch gel electrophoresis, immunoelectrophoresis, chromatography on Sephadex G 200, immunoprecipitation with specific antisera and precipitation with ammoniumsulphate and trichloroacetic acid. The third serum *protein fraction was characterized by starch gel electrophoresis as an alpha-one globulin migrating* immediately behind albumin By gel filtration it was eluted with albumin and it was soluble in 80 ammonium sulphate, but completely precipitated by trichloroacetic acid and by Rivanol. The proportion of the radioactivity incorporated into the three fractions was independent of the amount of insulin present but proportional to the duration of the incubation. The results indicate that the incorporation of radioactivity into the alpha-2 M and alpha-one globulin continues throughout the incubation period with a constant rate of 1 and 0.5 per cent per 24 hrs respectively except for a rapid initial association of approximately 4 per cent to the alpha-one globulin. In contrast, the incorporation into the beta 2 M globulin reaches a constant level within the first week. About 10 per cent of the radioactivity in the macroglobulin fraction and 32 per cent of that in the alpha-one globulin fraction could be precipitated by immunoprecipitation for insulin.

The origin and physiologic significance of the insulin like activity (ILA) of serum, measured by the *in vitro* technique with rat adipose tissue has been debated. Since the properties of ILA differ in immunologic, biochemical and biological respects from crystalline insulin the contentious issue is whether ILA represents an association between insulin and serum proteins, or it is of unspecific nature, unrelated to the pancreas and to the regulation of metabolism (Gjedde 1964 Berson and Yalow 1965).

In order to investigate a possible association between insulin and normal serum proteins, radioactive labelled insulin has to be used in order to isolate and define chemically the complex by methods independent of its biological properties, i.e. insulin-like

activity Experiments of this type have been performed in different ways, first by Kallee 1952, who used paperelectrophoresis for the separation of insulin and serum proteins. Berson *et al* (1956) found, however that radiation damaged insulin behaves differently from native insulin on paperelectrophoresis, and they concluded that protein bound radioactivity appearing after incubation of serum with radioactive insulin represents damaged insulin" and that the experiments with labelled insulin therefore are so difficult to interpret that they must be considered a regrettable waste of effort (Berson and Yalow 1965)

However a binding of native insulin to normal serum proteins cannot be definitely disproved in this way and the question must therefore still be considered unsettled To differentiate between incubation artifacts and the formation *in vitro* of a possible physiologic complex it is necessary to identify the products by several different separation procedures including immunological reactions and then compare such products with serum proteins which exhibit insulin like activity The association *in vitro* between insulin and serum proteins might involve slow and irreversible processes, so that prolonged periods of incubation has to be employed in order to reveal such associations.

In the present investigation experiments of this kind have been performed with ^{131}I labelled homologous insulin of moderate specific activity They are extensions of an earlier investigation in which it was demonstrated that two normal serum proteins become labelled when serum is incubated for several days with labelled insulin (Clausen, Gjedde and Jorgensen 1963)

Material and methods

Insulin ^{131}*I* Crystalline insulin of human or feline origin was labelled with ^{131}I by iodination in an acid medium (K. Jorgensen 1966 A and B) The specific activity of the preparations varied from 5 to 15 mC per mg and the degree of iodination was less than one iodine atom per 10 insulin monomers. The insulin was dissolved t concentration of 60 μC per ml in phosphate buffer 40 mM, pH 7 4 which also contained bovine serum albumin, 0.1 %, merthiolate 0.01 % and NaCl 0.9 %. This solution was stored t — 22° C. The radioactive purity was ascertained by the following criteria

1 Human and feline ^{131}I labelled insulin was diluted with carrier mount of pork insulin and subjected to starch block electrophoresis nd chromatography on Sephadex *G* 200. The specific activity remained consta t through the whol protein fraction nd showed only one single peak (Fig 1)

2. 100 of the radioactivity could be precipita ed with trichloroacetic acid (TCA) to final *concentration of 5 % (W/V) when pork insulin was dded t final concentration of 1—5 mg pro*tein per ml.

3 Bioassay using the adipose tissue or rat hemidiaphragm techniqu showed the biological activity of the ^{131}*I labelled insulin be undistinguishable from unlabelled insulin.*

4 95—98 % of th radioactivity could be precipit ted by the following immunological procedure (double immunoprecipitation) which is modification of the method described by Morgan and Lazarow (1962) 100 μl of 2 % gui ea-pig anti-insulin serum (*GPAIS*) in 10 *mM* phosphate — 0.9 % NaCl buffer pH 7 4 capable of neutralizing 1 000 microunits of insulin was added to 1 ml of the Sephadex separated fractions, or 10 μl of serum, diluted to 1 ml with the same phosphate buffer Af er 24 hours t 4 C, 100 μl of rabbit ti-(guinea-pig globulin) serum (*RAGPS*) as added, and the mixture was left for 4 hrs t 4 centrifuged at 4 (2,500 rpm) and washed two times with 200 μl of cold phosphate-saline buffer The minimum yield of labelled insulin was 95 % of the TCA precipitabl activity independent of the initial specifi activity of insulin.

Guinea-pig anti-insulin serum (GPAIS) was prepared by injecting guinea-pigs with 1 2 (V/V) *mixture* of pork or ox insulin (Insulin Semilent or Lent) and emulsio paraffinl (Pharmacopoea Danica) The dose was 10 to 20 units subcutaneously once weekly for 10 weeks.

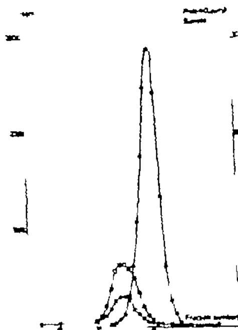


Fig 1 Control of radioactivity purity Gel filtration on Sephadex G 200 of mixture of ^{131}I labelled human insulin and 5 mg pork insulin. Protein (Lowry Units): (□), radioactivity (cpm) (●) and sucrose (anthrone) (○) The specific activity remains constant through the protein fractions and there is only one single peak of radioactivity Sucrose, used as an indicator of the total gel volume, is eluted together with and immediately after the insulin.

Rabbit anti (guinea-pig globulin) serum (RAGPS) Saturated ammonium sulphate was added to serum from normal guinea-pigs to 40 saturation. The precipitate was dissolved by addition of distilled water to the original serum volume and reprecipitated twice After dialysis against saline the globulins were mixed with equal volumes of emulsio paraffin in Waring blendor and 2 ml of the emulsion was injected subcutaneously once weekly for 8 weeks

Immunoprecipitation of alpha-2 M and beta-2 M were performed with rabbit anti-(human alpha-2 M) and rabbit anti-(human beta-2 M) sera, purchased from Behringwerke, AG, Marburg-Lahn, Western Germany or from the Central Laboratories, The Blood Transfusion Service Netherland Red Cross, Amsterdam, Holland 10 µl of whole human serum in 1 ml phosphate saline, buffer pH 7 4 or 1 ml fractions of Sephadex fractionated human serum were titrated to maximal precipitation of radioactivity After 24 hrs at 4 C the mixture was centrifuged at 4 and the precipitate was washed twice with 200 µl cold phosphate-saline buffer at pH 7 4.

Starch-block electrophoresis was performed by the method of Kunkel (1932) using phosphate buffer 20 mM pH 7 4 and 500 V 35 mA for 20 hours at 4

Horizontal starch-gel electrophoresis was carried out after the method of Smithies (1959), using Poulik (1957) discontinuous Tris-citric acid borate buffers After electrophoresis at 4 for 18 hrs at 100 to 150 V and 26 to 30 mA the gel was cut horizontally and the upper half stained with Amidoblack 10 B in order to locate the protein bands

The lower half was cut into sections, corresponding to the various protein fractions after correction for the shrinkage caused by the staining procedure and the radioactivity of the sections was measured

Sephadex G 200 The columns were prepared by pouring the gel into vertically suspended glass columns 2 2 60 cm, with 5 mm thick layer of Sephadex G 25 (coarse) layered over coarse sintered glass filter in the bottom. The void volume of the columns was about 50 ml and the total volume about 200 ml 1 ml serum was applied to the columns. The columns were eluted at room temperature with 10 mM phosphate buffer pH 7 4, containing 0.9 NaCl and 0.01 merthiolate. The columns did not contain residual radioactivity after repeated runs and the recovery of added labelled insulin was 97 with coefficient of variation of 2 (in 6 expts). Protein was determined by Lowry method (Daughaday et al 1952), and sucrose by the anthrone method (Hamid and Abraham 1957) as modified by Gjedde (unpublished)

Radioactivity was determined in Frieseke Hoepfner well-type scintillation counter By using channel analyser, the background was reduced to 5 per cent, with concomitant fall of the counting efficiency of 40 per cent

Procedure of incubation of serum with radioactive insulin Blood was drawn from healthy fasting volunteers into chilled tubes and immediately centrifuged, coagulation being thereby prevented. Plasma

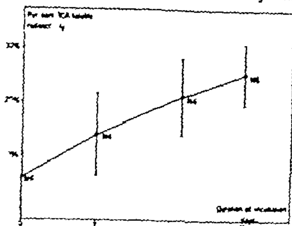


Fig 2. Trichloroacetic acid soluble radioacti ity in per cent of total serum radioactivity after different periods of incubation. Mean ± 1 S.D N number of in estigations.

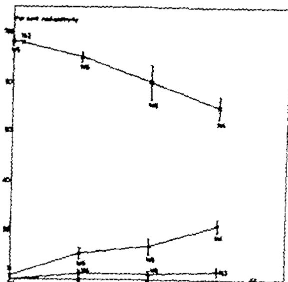


Fig 3 Radioacti y precipitated from whole serum by specific antisera against huma alpha-2 M (●) and beta-2 M ( ) and by double immunoprecipitation of insulin (□) in per cen of trichloroacetic acid precipitable radioactivity Mean ± 1 S.D N number of in estigations

was removed, nd allowed t clot. Radioactiv insulin in volume from 100 to 500 µl, orresponding to 400 to 10 000 microunits per ml serum, 100 µl 1 sodium merthiolat and 1 g "bacteriological" sucrose was mixed with 10 ml serum The sera were kept at 22 C in gas phase of 95 oxygen and 5 carbon dioxide in order maintain pH of 7 4 Fi out of six bacterial cultures of serum samples after 7 to 21 days of incubation ere completely negativ. One culture showed few colonies of gramnegative rods The inoculation of sera were performed ith 20 fold dil tion of the samples.

Dialysis. as performed ith Visking seamless ellophane tubing 18/32, previously heated at 70—80° C for 2—4 hours in the medium employed for the dialysis

Results

When serum is incubated with iodinated insulin of moderate specific activity some radioactivity appears which can not be precipitated with trichloroacetic acid, indicating the formation of degradation products (Fig 2) Simultaneously a decrease of the radioactivity precipitated with the double immunoprecipitation for insulin

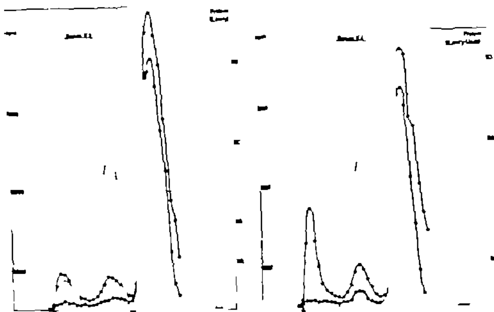


Fig. 4. Gel filtration on Sephadex G 200 of serum, containing 10,000 microunits ^{131}I labelled human insulin per ml serum, after incubation for 7 (E2) and 21 (E 4) days respectively. The first radioactivity peak corresponds to the macroglobulins, the second to the alpha-one globulin, and the third to free insulin and radioactive degradation products of low molecular weight. Note that more than 10 per cent of the radioactivity of the macroglobulin fraction and more than 30 per cent of the radioactivity of the alpha-one globulin is precipitated by double immunoprecipitation for insulin. (●): radioactivity of the fractions, ( ) radioactivity of the precipitates after double immunoprecipitation for insulin, ———— protein in Lowry units.

is observed. That the latter decline is related to an association of radioactivity with serum proteins and not necessarily to degradation was brought out in the following experiments.

Serum samples incubated for various periods with ^{131}I labelled insulin were treated with specific antisera against human alpha 2 M globulin and against human beta 2 M globulin. The radioactivity of the precipitates demonstrated binding of radioactivity to these macroglobulins, especially to alpha-2 M globulin, which had 5 times the activity found with beta 2 M-globulin (Fig. 3).

When sera incubated for 7 days were subjected to Sephadex G 200 chromatography, the radioactivity was separated into three peaks, the first corresponding to the macroglobulins (Flodin and Killander 1962), the second located together with the main protein peak, and the third and largest appearing after nearly all the protein had been eluted (Fig. 4. Serum E 2). When the same separation was performed on sera incubated for 21 days a marked increase of the amount of radioactivity of the macroglobulin fraction was observed (Fig. 4. serum E 4).

In order to follow the rate of incorporation into the different fractions, fractionation on Sephadex G 200 of the same sera incubated for periods from less than 10 min to 21 days was performed. Fig. 5 shows the figures for incorporation of radioactivity

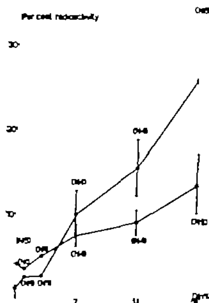


Fig 5. Accumulation of radioactivity in the macroglobulin, (●) and I the alpha-one globulin fraction (○) of serum, separated on Sephadex G 200, after different periods of incubation, expressed as per cent of total eluted radioactivity Mean ± 1 S.D N number of investigations.

T ɪʟᴇ I *Per cent of radioactivity in serum fractions, separated by Sephadex G 200 filtration, precipitated with addition of mmonium sulpha t 80 per cent saturation at pH 8 0. Th sera had been ncubated from 1 to 21 days with 10,000 microunits ¹³¹I labelled homologous insulin*

	Per cent precipitated
Macroglobulin fraction from human serum	
Mean ± 1 S.D	93 ± 3 4 per cent (N 7)
Macroglobulin fraction from feline serum	
Mean ± 1 S D	95 ± 3.5 per cent (N 5)
Alpha-1 1 fraction from huma serum	
Mean ± 1 S.D	10.6 ± 2.6 per cent (N 7)
Alpha 1 1 fraction from feline serum	
Mean ± 1 S D	10.7 ± 1.2 per cent (N 5)
Free insuli fraction from human serum, added carrier insuli 1 mg/ml	
Mean ± 1 S D	59 ± 13 per cent (N 5)

into the macroglobulin and the middle fraction The proportion of radioactivity incorporated into the two fractions was independent f the amount of insul n present and proportional to the duration of incubation. It is seen that the values for the association of radioactivity with the macroglobulin fraction are in good agreement with the data found by precipitation with specific antisera (Fig 3)

The third and largest fraction of radioactivity of gel filtrated serum comprised free radioactive insulin together with radioactive degradation products. 60 per cent

TABLE II Starch gel electrophoresis of serum after incubation with 10,000 microunits ^{131}I labelled homologous insulin for 7 days. The radioactivity in the fractions are expressed as per cent of total recovered radioactivity. Mean ± 1 S.D.

	Beta-2 M	Alpha-2 M	Alpha 1 1
Human Serum C	1.5 (N 1)	12.3 % (N 1)	7.3 (N 1)
Human Serum E	2.4 ± 1.2 (N 4)	9.2 ± 0.7 % (N 4)	8.5 ± 0.4 % (N 4)
Feline Serum no. 2	3.1 ± 0.6 % (N 4)	16.8 ± 2.3 % (N 4)	10.5 ± 1.2 (N 4)

TABLE III Per cent of radioactivity of serum fractions, separated by Sephadex G 200 filtration, precipitated with double immunoprecipitation for insulin.
Mean ± 1 S.D. N number of investigations.
The sera had been incubated with 10,000 microunits ^{131}I labelled insulin for periods from 10 min to 21 days.

Macroglobulin fraction	10.2 ± 3.1 % precip. (N 9)
Alpha-one-one globulin	32 ± 5.7 precip. (N 9)
Free insulin fraction	81 ± 10.6 % precip. (N 9)

of this radioactivity could be precipitated by 80 % saturation with ammonium sulphate after addition of carrier insulin 1 mg per ml, where in control experiments 90 per cent of radioactive insulin was precipitated (Table I).

When the fractions of gel filtrated sera were subjected to electrophoresis in starch gel a further characterization was obtained. The radioactivity of the macroglobulins separated into two bands, corresponding to beta 2 M and to alpha 2 M (identical with alpha 2-5 Smithies 1959). Gel electrophoresis of the middle fraction showed that the radioactivity was associated with an alpha-one globulin in the following called alpha-one-one globulin, located immediately after albumin.

By starch gel electrophoresis of whole serum the rate of incorporation of radioactivity into the three protein fractions was confirmed (Table II).

In order to characterize the radioactive alpha-one-one globulin, the solubility properties of this component was examined. It was found to be completely precipitated with trichloroacetic acid (final concentration 5 g per 100 ml) or with Rivanol (final concentration 5 g per 100 ml) or with Rivanol (final concentration 0.4 g per 100 ml) but only about 11 per cent of the radioactivity was precipitated by the addition of ammonium sulphate to 80 % saturation (Table I).

By double immunoprecipitation with anti-insulin serum, 10 per cent of the Sephadex separated macroglobulin radioactivity 32 per cent of the alpha-one-one and 81 per cent of the "free insulin" radioactivity were precipitated (Table III) indicating an association with serum proteins of radioactive components with insulin structure. Control experiments for coprecipitation were performed by adding normal guinea-pig serum instead of anti-insulin serum to the Sephadex separated fractions; less than 2 per cent of the radioactivity was found in the precipitates.

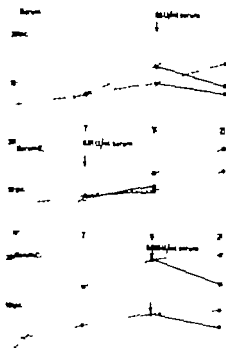


Fig 6 Reversibility of the association of radioactivity to the macroglobulin (□) and t the alpha-one globulin (●) *fraction of serum shown by incubation* with unlabelled insulin. The sera had been preincubated with 10,000 microunits ¹²⁵I insulin and unlabelled insulin was added as ndicated. Samples ere taken t 7 days intervals for Sephadex G 200 filtration. The dotted line indicates the accumulation of radioactivity in the two fractions in the bsence of unlabelled insulin. The full line denotes the change of th mount of radioactivity in the two fractions after addition of unlabelled insulin.

TABLE IV Accumulation of radioactivity in the macroglobulin and i the alpha-one globulin fraction of human and feline serum, separated on Sephadex G 200, expressed as per cent of total eluted radioactivity Mean ± 1 S.D

Human serum	0 days. (less than 10 min.)	7 days.	14 days.
Macroglobulin	1 17 ± 0 41 % (N 5)	10.1 ± 2.7 % (N 4)	15.5 ± 3.2 (N 4)
Alpha-one-one glob	4 05 ± 1.90 % (N 5)	7.6 ± 2.0 (N 4)	9 0 ± 1.6 % (N 4)
Feline serum	1 day	7 days.	
Macroglobulin	4.5 ± 1 4 (N 2)	17.5 ± 5.0 % (N 2)	
Alpha-one glob.	9.5 ± 0.9 % (N 2)	11.9 ± 0.7 % (N 2)	

Reversibility of the binding of radioactivity was investigated by adding unlabelled human insulin to sera, which had been incubated with 10 000 microunits ¹²⁵I human insulin per ml for 2 weeks. Further incubation for a week with a vast excess of unlabelled insulin, 500 000 to 635 000 microunits per ml serum reduced the binding to the macroglobulin and the alpha-one-one globulin fraction to the same level as that obtained 7 days before the addition (Fig. 6)

Radioactive cat insulin, added to serum from normal cats, showed binding to the same serum fractions as human insulin both by separation on Sephadex G 200

and on starch gel electrophoresis. Moreover the same percentage of radioactivity in the three Sephadex separated fractions of feline serum was precipitated with immunoprecipitation of insulin as that of human serum. It was apparent, however that nearly the double amount of radioactivity was accumulated in the macroglobulin and the alpha-one-one globulin fractions after incubation for 7 days compared with human sera (Table IV)

To test the possibility that dialyzable degradation products of radioactive insulin were incorporated into serum fractions, 5 ml of human serum, incubated for 21 days with 10 000 microunits ^{131}I human insulin and containing 30 per cent of its radioactivity in dialyzable form, was dialyzed against 10 ml destilled water for 24 hrs, whereafter the dialysat was evaporated at room temperature with a water suction pump, and 2 ml of fresh human serum was added. The mixture was left for seven days at 22 C and fractionated on a Sephadex G 200 column. Since all the radioactivity was eluted after the proteins, and since furthermore the radioactivity of the macroglobulin fraction and of the alpha-one-one globulin fraction from Sephadex G 200 separated human serum was not reduced after extensive dialysis against 20 volumes of Krebs Henseleit bicarbonate buffer containing 1 mM KI at 4 C for 24 hrs, it can be concluded that the presence of radioactivity in these fractions is not due to dialyzable degradation products of the labelled insulin.

In order to investigate the possibility that products formed from labelled insulin by radiation or incubation damage behaved as macroglobulins or alpha-one-one globulin in gel filtration, the following experiments were made. Pork insulin, to which labelled human or feline insulin was added, was incubated for seven days 22° C. Upon filtration on Sephadex G 200 the same radioactivity and protein pattern was found as before incubation. Likewise, ^{131}I labelled insulin stored at —22 C in serum for 42 days showed the same fractional incorporation after separation on Sephadex G 200 as before freezing

No difference with respect to association of radioactivity with serum protein fractions was observed when a mixture of penicillin and streptomycin was substituted for merthiolate in the incubation mixture. This was also the case in a few experiments where no bactericidal agent was added at all.

Discussion

The results of earlier investigations of the question whether or not insulin *in vitro* is bound to serum proteins are contradictory Using paperelectrophoresis, and tacitl assuming that a possible exchange between labelled and unlabelled insulin is rapidly reversible, it has been claimed that insulin is not bound to serum proteins (Berson et al. 1956, Burrows, Peters and Lowell 1957 Kalant, Gomberg and Schucher 1958 In contrast, other investigators, using electrophoresis on paper resin paper or agar gel have maintained that very large amounts of radioactive insulin became associated with serum proteins (Duncombe and Mann 1962, Mitchell 1960 Prout *et al* 1963)

The observation (Clausen, Gjedde and Jorgensen 1963) that radioactivity is primarily bound to alpha 2 M globulin, when normal serum is incubated with labelled insulin, has been confirmed by others Zahnd and Scheidegger 1963 Kallee, De blasi and Addabo 1963 Gerling and Sirek 1965 Berson and Yalow 1965 However the latter authors consider the labelling of the serum protein fractions to be caused by radioactive degradation products derived from incubation or radiation damage of the labelled insulin. That the association of radioactivity with the three serum proteins fractions, demonstrated in the present investigation, is due to dialyzable degradation products seems to be ruled out, since it was also shown that such compounds did not cause labelling of serum proteins. The presence of radioactivity in the serum proteins must therefore signify an association with either denatured or native labelled insulin. That minor fractions of the labelled macroglobulins and alpha-one-one globulin are precipitated by a double immunological reaction against crystalline insulin is compatible with both possibilities, since it is well known that a denatured protein can maintain its immunological reactivity and a distinction between the two possibilities has to be based on other biological properties of the different fractions. A further discussion of this question will therefore be presented in connection with the studies of ILA of different serum protein fractions (Gjedde, in preparation)

The dominant feature of the association of radioactivity from labelled insulin with the three serum proteins is the slow rate with which the incorporation of radioactivity and the exchange with unlabelled insulin takes place. This is in contrast to the rapidly reversible association between thyroxine and plasma proteins (Daughaday 1960) and between cortisol and transcortin (Ingbar and Freinkel 1960)

However a discussion of the slow rate of association between radioactivity and the three serum proteins when serum is incubated with labelled insulin can only be speculative, as long as the nature of the association and the exact identity of the radioactive reactants are unknown.

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The Effect of Thyroidectomy on the Hypothalamus of the Rat with Special Reference to the Hypothalamic Hypophyseal Neurosecretory System

By

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Abstract

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With male adult albino rats for experimental animals, th effects of operative thyroparathyroidectomy and of thyroidectomy accomplished with radioactive iodine on th nuclear volume of the ganglion cells in the nuclei of the hypothalamus were studied. I addition, the mount and distribution of aldehyde-fuchsin-positive neurosecretory material under these experimental conditions were studied. The changes were similar in rats ectomized by either procedure I comparison to untreated control animals, the nuclear volume of the ganglion cells decreased in all nuclei investiga ed. The change was statistically highly significant in most instances. The amount of neurosecretory ..ial seemed to decrease, on the strength of visual assessment both in the neurohypophysis and the median eminence. The depletion of the material in the neurohypophysis could also be demonstrated by photometric measurement. From the results the inference was drawn that decrease in the activity of the hypothalamus occurs after thyroidectomy At the same time also the activity of the eurosecretory system goes down, which is reflected by depletion of the neurosecretion N sharply localized regulatory centre could be shown for the pituitary-thyroid interplay because th entire hypothalamus reacted in identical manner

The histo-physiological changes caused by thyroidectomy in the hypothalamus have been studied in the attempt to localize that part of the brain which acts as a potential regulator of the secretion of thyroid-controlling thyrotropic hormone. The existence of such a hypothalamic centre is suggested by numerous physiological and biochemical studies (for references, see *e g* Szentágothai *et al.* 1962 D'Angelo 1963) It has to be noted, however that the link between the hypothalamus and the pituitary-thyroid interplay is still poorly understood in its character It is thought that both neural and humoral mechanisms are associated with it.

Morphological studies carried out so far with reference to the changes produced by thyroidectomy in the hypothalamus deal mainly with neurosecretory system (Shibusawa *et al.* 1956 Shiozaki 1956 Matsui and Engelhardt 1960 Ford 1960).

To some extent also karyometric measurements of ganglion cells in the nuclei of the hypothalamus have been performed (Mess and Kutas 1959, Matsui and Engelhardt 1960, Talanti 1965), but far from all nuclei have been covered by karyometry in such activity determinations. The results obtained in the few existing studies are rather conflicting.

The principal aim of the present work was to subject to karyometric measurement the changes observable after thyroidectomy in all the commonest nuclei of the rat's hypothalamus. It was to be an attempt to find such an area or areas where the changes in nuclear volume of the ganglion cells would differ from the corresponding changes elsewhere in the hypothalamus. Such areas might constitute localized regulatory centres for the function of the pituitary-thyroid axis. In addition, the purpose was to study the quantity and distribution of neurosecretory material in the rat's hypothalamic-hypophyseal neurosecretory system after thyroidectomy.

Material and methods

Thirty male albino rats weighing 181—234 g were used. They were fed a basic standard pellet diet of constant composition *ad libitum*. Tap water was available in unrestricted amount. All rats were kept in the same room, where the temperature varied within 21.0—23.5° C during the test. The animals of the radiothyroidectomy group constituted an exception in that they were kept for 3 days after radio-iodine administration in a separate room in conditions consistent with those of the experimental animal room proper. The animals were divided into groups which are presented in Table I.

The operative thyroidectomy was in fact thyroparathyroidectomy. It was performed in Nembutal narcosis. Both macroscopic assessment and examination at autopsy of sections made with the cryostate microtome from the tissue in the laryngeal region showed that the operation had been successful. Radiothyroidectomy was produced by intraperitoneal administration of ^{131}I in a quantity equivalent to 1.5 mC per animal. Originally ten animals were thus treated, but macroscopical examination at autopsy revealed that complete radiothyroidectomy had only been achieved in six animals, which were taken into account in the results. The control animals numbered initially 15, but one of them died during the test period. The cause of death could not be elicited at autopsy. Both test groups had their own control group. The administration of radioiodine in the radiothyroidectomy group and the operative interference in the group of operative thyroidectomy were performed in the course of one day to all animals. The ectomized animals were sacrificed eight weeks later, on the same day as the control animals. Sacrifice was by rapid decapitation, and all animals were killed within one hour.

TABLE I. Body weight, weight of the hypophysis, protein-bound iodine (PBI) of the plasma and serum cholesterol of the experimental animals.

Group	Number of animals	Mean body weight, g ± S.E.M. Initial	Final	Mean weight of the hypophysis, mg/kg b.w. ± S.E.M.	Mean PBI µg/100 ml	Mean cholesterol mg/100 ml
Operative thyroidectomy	10	209 ± 12	221 ± 11	62.1 ± 4.3	1.2	53
Controls	8	214 ± 9	267 ± 10	34.4 ± 2.5	6.4	[illegible]
Radio-thyroidectomy	6	214 ± 12	229 ± 10	60.9 ± 5.1	1.9	1.2
Controls	6	218 ± 11	278 ± 12	36.2 ± 2.4	[illegible]9	

TABLE 11 Results of karyometric measurements. Cross section areas of the ganglion cell nuclei stated as percentages of the corresponding values found for the control animals. (Mean ± S.E.M.)

Hypothalamic nucleus	Operative thyroidectomy Controls	Ectomized	P	Radiothyroidectomy Controls	Ectomized	P
Area preoptica	100 ± 1.06	87.92 ± 1.01	< 0.001	100 ± 1.22	89.93 ± 1.50	< 0.001
Ncl. suprachias-						
maticus	100 ± 0.98	79.86 ± 1.42	< 0.001	100 ± 1.00	94.70 ± 1.36	< 0.01
terior	100 ± 1.28	86.05 ± 1.11	< 0.001	100 ± 1.70	93.01 ± 1.56	< 0.001
» upraopticus	100 ± 1.23	93.73 ± 1.20	< 0.001	100 ± 1.47	91.39 ± 1.48	< 0.001
» para entricularis	100 ± 1.55	78.64 ± 1.54	< 0.001	100 ± 1.20	93.01 ± 1.14	< 0.001
infundibularis	100 ± 0.90	92.08 ± 0.89	< 0.001	100 ± 1.09	96.20 ± 1.24	< 0.05
» ventromedialis	100 ± 1.00	92.30 ± 0.93	0.001	100 ± 1.14	93.38 ± 2.17	< 0.01
dorsomedialis	100 ± 1.18	97.48 ± 1.28	> 0.05	100 ± 1.64	94.71 ± 1.31	< 0.05
Subst. periventri-						
cularis	100 ± 1.95	92.44 ± 1.80	0.001	100 ± 1.13	84.53 ± 1.38	< 0.001
Area dorsalis	100 ± 0.64	92.96 ± 1.79	< 0.001	100 ± 2.05	94.1 ± 2.02	< 0.05
dorsocaudalis	100 ± 2.20	79.95 ± 1.39	< 0.001	100 ± 2.50	92.73 ± 1.66	< 0.01
lateralis	100 ± 1.69	97.44 ± 1.89	> 0.05	100 ± 2.13	97.13 ± 2.58	> 0.05
Ncl. premamillaris	100 ± 1.08	93.17 ± 1.00	< 0.001	100 ± 1.54	94.73 ± 0.96	< 0.01
supramamillaris	100 ± 1.62	80.92 ± 1.22	0.001	100 ± 2.01	96.47 1.64	> 0.05
mamillaris med	100 _ 0.98	76.83 ± 0.92	0.001	100 ± 1.36	91.28 ± 1.61	< 0.001
mamillaris lat	100 ± 1.27	94.30 ± 1.30	0.01	100 ± 04	82.38 ± 0.91	< 0.001

The brains and hypophyses were embedded in paraffin after fixation 10 formalin. The brains were sectioned in the sagittal plane serial sections 10 . Every fourth section as chosen for examination. Of them, every second was stained by haematoxylin-eosin nd every second by Gomori aldehyd -fuchsin according to Landing, Hall nd West (1956). Th hypophyses, too, were processed int serial sections. Thei major was stained h aldeh de-fuchsin add the rest according t Halmi (1952) method for pit ary basophils.

For determination of the nuclear cross section area of ganglion cells, mage of he uclear as projected 1 700 magnification on drawing board by th aid of amer lucida and contour as traced on paper. The re s ere then measured by planimetry. The uclea re of bout 50 ganglion cells of each hypothalamic nucleus to be nvestiga ed was measured each animal. I all bout 24 000 cells ere measured.

The amount of neurosecretory ma erial in differen parts of the h pothalamic h pophyseal neurosecretory system was recorded on the basis of sual assessment and, in the eurob pophysis, also by photometric measurements. The sual assessments were aimed reflect he mount of stained material nd scoring system from 0 to 5 as employed. Such sual assessmen as made five times and the mean as taken of these replicate assessments. Photometric measurement of the selectively tainabl material in th neurohypophy as performed by the aid of Leitz MPE (STG 12) microphotometer using 4 objective lens and 530 μ fil er. For each section from the neurohypophysis th quantity of transmitted ligh was determined t not less than 20 points. I evaluating the results, the quantity of ligh bsorbed was assumed be direc l proportional to the amount of darkstained neurosecretory material i the specimen. N measurements ere made of the thickness of the sections.

The sections from differen experimental groups between which omparison ith regard to amount of neurosecretory material was desired were al y stained simultaneously in he same reagent and staining solutions. This was done in order t make the staining conditions as closely identical as possible.

From the series of observations obtained, the means nd their standard errors ere alculated. Student t test was pplied in comparing means (Fisher 1950).

TABLE III Estimated amount of aldehyde-fuchsin-positive neurosecretory material in different

Group	I.P		M.E.	
	Mean ± S.E.M.	P	Mean ± S.E.M.	P
Operative thyroidectomy	1.7±0.1	<0.001	2.4±0.2	<0.001
Controls	4.1±0.2		4.0±0.2	
Radiothyroidectomy	1.5±0.1	<0.001	2.9±0.2	<0.001
Controls	4.3±0.2		4.3±0.2	

I.P = Infundibular process, M.E = Median eminence H.H.T = Hypothalamic-hypophyseal

Results

Karyometric measurements of the hypothalamic nuclei. — The data are stated in Table II. The results have been given, for greater clearness, in per cent with reference to the value found for the control animals of each group

The cross section area of the nuclei decreased after thyroidectomy in all nuclei. This same tendency was observed after operative as well as radiothyroidectomy Statistically highly significant differences were established in many of the nuclei.

The distribution graphs of the nucleus cross section areas had largely similar shape in the test and control groups corresponding to each other for instance, the ranges were equal. The sole difference observable in the graphs was their shift towards smaller values in the thyroidectomy groups.

Aldehyde-fuchsin positive neurosecretory material. — The data representing the visual assessments are stated in Table III The results of photometric measurements relating to the neurohypophysis can be seen in Table IV

The results indicate that the amount of neurosecretory material decreased at a statistically significant level in the distal part of the neurosecretory system on thyroidectomy The diminution was greatest in the neurohypophysis where the material is encountered in highest quantity Also in the median eminence a statistically significant difference was established, whereas no statistically significant differences were elicited in the parts of the system proximal to it. The variation in amount of neurosecretory material between ganglion cells seemed to be greater in the thyroidectomized animals than in the controls. The over all impression was gained that the material in the neurosecretory nuclei was slightly depleted, but this tendency did not amount to a statistically significant difference either

Adenohypophysis — The cytological picture was characterized by virtually complete disappearance of the acidophils and an increase of large multi-vacuolated basophils with aldehyde fuchsin-positive granulation. The cells of the latter kind were thyroidectomy cells.

Discussion

The karyometric results of the present study are in agreement with the previous results of Matsui and Engelhardt (1960) who performed measurements upon operative thyroidectomy in the rat's neurosecretory nuclei and *in its infundibular*

parts of the hypothalamic hypophyseal system.

H.H.T Mean ± S.E.M.	P	P.V.N Mean ± S.E.M.	P	S.O.N Mean ± S.E.M.	P
3.7±0.2	>0.05	3.6±0.3	>0.05	4.2±0.2	>0.05
4.0±0.3		3.9±0.3		4.5±0.2	
3.9±0.2	>0.05	3.7±0.2	>0.05	4.0±0.2	>0.05
4.2±0.2		4.2±0.2		4.4±0.2	

tract, P.V.N = Para ventricular nucleus, S.O.N = *Supraoptic nucleus*

TABLE IV. Results of photometric measurements of the aldehyde-fuchsin-stained sections from the neurohypophysis. Percentages of transmitted light.

Group	Mean ± S.E.M.	P
Operative thyroidectomy	34.2±1.0	<0.001
Controls	25.7±0.7	
Radiothyroidectomy	36.1±1.1	<0.001
Controls	24.8±0.6	

nucleus, ventromedial nucleus and dorsomedical nucleus. The results of karyometric measurements on the neurosecretory nuclei made previously by the author (Talanti 1965) too are similar to those gained in the present work. Comparison with the results of Shibusawa *et al.* (1956) is difficult because these authors do not state the actual results of measurements. They merely state that the nuclear volume of ganglion cells in the neurosecretory nuclei increase after thyroidectomy. Mess and Kotor (1959) and Szentágothai *et al.* (1962) obtained results somewhat at variance with those recorded in the present instance. In their material the nuclear size of ganglia in the paraventricular nucleus increased, whereas that in the premamillary nucleus became less. In respect of the other nuclei investigated no statistically significant difference between the test and control groups could be established.

It is rather difficult to account for the contradiction with earlier results. They are obviously largely due to differences in experimental conditions and methods of measurement. For instance another length of a experimental period was used at least in part of the previous studies. The same applies to the procedure of karyometric measurement.

The earlier results of investigation concerning the effects of thyroidectomy on the amount of neurosecretory material are somewhat conflicting. This is readily understandable, seeing that objective measurement of the quantity of neurosecretory material poses considerable difficulties. Shibusawa *et al.* (1956) observed a decrease of the amount of neurosecretion not only in cells of the neurosecretory nuclei but also along the entire supraoptico-hypophyseal pathway especially in the neuro-

hypophysis. They attribute a decrease in the amount of neurosecretion to a rise in activity of the neurosecretory system. According to Shiozaki (1956) total thyroidectomy initially causes an accumulation of neurosecretory material in the neurohypophysis and also in the cytoplasm of the ganglion in the paraventricular nucleus. However at the end of 40 postoperative days the material was depleted from the neurohypophysis even to such an extent that the amount present was far less than the average amount in normal animals. In the ganglion cells of the supraoptic nucleus, on the other hand, Shiozaki noted no changes after thyroidectomy. Matsui and Engelhardt (1960) found that a decrease in the amount of neurosecretion occurred in the neurohypophysis and in the infundibular area, whereas it was even increased from normal in the proximal part of the neurosecretory system. According to Ford's (1960) studies the neurosecretion in the supraoptic and paraventricular nuclei and axons was observed to be increased by thyroidectomy. Neurohypophyseal changes were minimal. This was interpreted to mean that there was increased output of neurosecretion without storage. The only change in the neurosecretion observed by Arko, Kivalo and Rinne (1963) in thyroidectomized rats 2—3 weeks after operation was a slight increase in the amount of the neurosecretory material around the portal capillaries in the median eminence.

The contradictions of the results are thought to arise at least in part from the factors introduced by visual estimation. The other factors responsible may also be differences in the staining method and in the experimental period. Aldehyde-fuchsin yields a better contrast than chrom-haematoxylin. In addition, the latter also stains Nissl's substance and this may affect the assessment of the neurosecretion quantity. The ether anaesthesia applied in some studies in association with decapitation may also affect the amount of neurosecretion (Rothballer 1956).

In many of above-reviewed previous studies in which the amount and distribution of neurosecretion in the hypothalamic hypophyseal system have been investigated, the results have been interpreted as indicating activation of the system. Considering the reduction of nuclear volume in the neurosecretory ganglion cells observed in this study it would seem logical that a decrease of the amount of neurosecretion after thyroidectomy is called forth by inactivation of the secretion. A decrease in activity was parallel in both neurosecretory nuclei and no bipartition of the mode of reaction, such as was previously reported (Shiozaki 1956), could thus be noted.

To be sure, what has been said above is valid on the specific assumption that the idea of changes in activity of the neurosecretory ganglia really being reflected by changes in nuclear volume is endorsed. In general, the nuclear size is a sensitive indicator of change in functional activity of many types of cells. Jacobj (1935) was the first to recognize the close connexion between nuclear size and cell function. Increased activity results in swelling of cell nuclei, while inactivity parallels with nuclear shrinking. However there are some exceptions to this rule in the case of exocrine as well as endocrine glandular cells (Salomon 1955, Halász 1958). Obviously the first to apply karyometry in investigation of the hypothalamus was Hertl (1952) who studied the changes during oestrus in the mouse's tuber cinereum. The method

has later been used in the study of neuroendocrine phenomena, particularly by a team of Hungarian investigators (for reference, see Szentágothai *et al.* 1962). The studies were especially aimed to localize the endocrine control functions in the hypothalamus and elsewhere in the central nervous system. In respect of neurosecretory cells it is to be observed that in them at least the secretory activity seems to be well reflected. As a sign of elevated secretion the nuclear volume increase, which is clearly observable in animals kept in thirst (e.g. Bachrach 1964).

When the reduction in nuclear volume occurring after thyroidectomy in the ganglion cells also of the other hypothalamic nuclei after thyroidectomy is taken into account, it is obvious that a decrease in activity of the ganglia of the neurosecretory nuclei is an indication of a decrease in hypothalamic total activity. Naturally karyometric investigation does not reveal in the slightest what kind of function it is that undergoes a change in activity. Excepting neurosecretory cells, little can therefore be said about the specific function of different nuclei, beyond this establishment of a decrease in activity. However, no specific hypothalamic centre regulating the pituitary thyroid interplay could be localized on the basis of the present study since the change observed by karyometry was general all over the hypothalamus. It would seem as if the entire hypothalamus as an entity participated in this regulation. This is the same conclusion which has been drawn by the Hungarian school (Szentágothai *et al.* 1962). The idea is fascinating indeed, considering that in likeness with other vegetative centres of the central nervous system, the hypothalamus is a rather primitive area of the brain, where the different functions are diffusely circumscribed. At any rate the result obtained contradicts earlier results of experiment according to which it was possible to localize the thyrotropin-regulating centre to a fairly small and demarcated area in the hypothalamus either by producing lesions (Greer 1951, Bogdanove and Halmi 1953, Ganong, Frederickson and Hume 1955, D'Angelo and Traum 1956) or by stimulation (Harris and Woods 1958, D'Angelo 1963).

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Heart Volume during Prolonged Exercise in the Supine and Sitting Position

By

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Abstract

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The roentgenological heart volume was studied in 10 healthy young men during prolonged continuous exercise of constant intensity in the sitting position and in 6 subjects also in the supine position. The work load was on an average 600.0 kpm/min in the sitting and [illegible] kpm/min in the supine position. The heart rate in the sitting position after 5—9 min exercise was 139.5 beats/min and 170 beats/min at the end of work, mean work time 56.5 min. The corresponding figures in the supine position were 127.8 and 1[illegible]6.7 beats/min, with a mean work time of 64.0 min. The exposure of the roentgen film was made in end-diastole. During the continuous exercise at a constant load there was a continuous fall of the heart volume in both body positions accompanied by a continuous increase in heart rate. There was a significant regression of total heart volume on heart rate. The rate of change in heart volume was 57.5 ml and 58.3 ml per 10 beats/min change in heart rate in the sitting and supine positions respectively. The maximum decrease was on an average [illegible] ml and [illegible] ml with an increase in heart rate of 28.6 beats/min and [illegible]4.8 beats/min in the sitting and supine position respectively. Close to exhaustion, the heart volume increased in 6 of 10 subjects in the sitting position by on an average [illegible]5 ml (probably significant) and in 3 of 6 in supine by on an average 4[illegible]0 ml in spite of a further increase in heart rate.

In earlier studies (Ekelund and Holmgren 1964, Ekelund 1966) the circulatory and respiratory adaptation during prolonged, non-steady state exercise was studied both in the sitting and in the supine position. Under both these experimental conditions there was a continuous fall of the stroke volume accompanied by a continuous increase in heart rate. Cardiac output was maintained constant. Central vascular pressure, i.e. the pressure in the central veins and arteries, fell at the same time. The observed changes might be explained by a shift of blood volume within the capacity vessels from the central circulation to the periphery. Such a shift should result in a decrease of the filling energy available to the heart and might cause the observed decrease in stroke volume.

The heart volume determined by roentgen examination during short periods of exercise of increasing intensity and thus with increasing heart rate, has been found

to vary in parallel with changes in stroke volum in normal subjects (Holmgren and Ovenfors 1960 Bevegård, Holmgren and Jonsson 1960) No tudies of the variations of the roentgenological heart volume during prolonged exercise seem to have been reported.

The present investigation was undertaken to study further the mechanisms responsible for the decrease in stroke volume observed during prolonged, non-steady state exercise. Under such experimental conditions one should expect a decrease in heart volume of a magnitude which is related to the expected decrease in troke volume, if impaired filling i a major determinant.

Material

The subjects consisted of ten healthy males of ordinary physical fitness, mean age 25.5 years (range 19—27) All were studied in the sitting position nd six both i the sitting and the supine position Prior to the investigation all subjects underwent clinical examination including chest X-ray ECG rest and during exercise heart and blood olume determinations, all of which were normal. One of the subjects also ook part in study of the central circulation with heart catheterization (Ekelund 1967) and wo took part i studies of the diffusing capacity during prolonged exercise (Ekel nd nd Holmgren 1965)

Methods

The methods employed for ECG-recording exercise esting, determination of heart olume in the prone position and total amount of hemoglobin, were the same as described in previous reports Ekelund nd Holmgren 1964 Ekelund 1966) Th determinations of heart olume during muscular work in the sitting and supine position ere performed with the aid of bi-plane serial film-changer (Elema)

Anteroposterior and lateral films were exposed simultaneously The exposures were made during the inspiratory phase, hich was determined with spirogram monitored on an oscilloscope The roentgen pparatus was triggered by the P-w ve of an oesophagus ECG The oesophagus ECG was recorded simultaneously with an indication of the duration of the exposures on two-channel direct-writing electrocardiograph (Fig 1) The exposure started after delay of, on an average 0 03 sec and maximally 0.04 sec. The exposure ime as 0 04 sec. Thus the exposure was completed 0 07—0 08 sec after the P-wav of he oesophagus ECG, see Fig 1

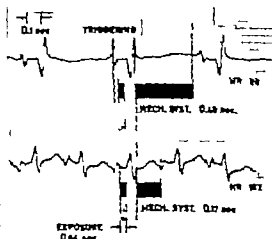


Fig 1 Time relationship between the exposure of the X-ra -films used for the determination of the heart volume during exercise and he electrical and mechanical systole t tw different heart rates (Beecher and Galetti 1963)

The focus-film distance was 1.0 m. The magnification was determined with the aid of [illegible] centimeter scale in the film plane (Larsson and Kjellberg 1948). The heart volume was determined by the formula of Kahlstorf for the ellipsoid (1932) as modified by Larsson and Kjellberg (1948) but with [illegible] constant correction factor for variations [illegible] the heart configuration. The coefficient of variation of [illegible] single determination estimated from duplicate determinations during exercise [illegible] 5.0 per cent in the sitting and 4.2 per cent in the supine position. The [illegible]alues for the heart volumes are given as the mean of 2—3 determinations made within one minute. The roentgen dosage [illegible] about 0.25 [illegible] per pair of exposures. The total roentgen dosage [illegible]aried bet[illegible]een 6—7 [illegible]

The statistical calculations were performed according t[illegible] Snedecor (1939) and Hald (1960).

Procedure

The experiments were first performed in th[illegible] sitting position. The investigations in th[illegible] supine position were performed two weeks t[illegible] tw[illegible] months later. The exercise was performed on an electrically braked ergometer (Holmgren and Mattsson 1954). I[illegible] the sitting position the height of the seat was adjusted so that the legs were almost completely extended with the pedals in the lowest position [illegible]nd the upper part of the body was held vertical and in [illegible] constant position by holding the hands on an adjustable support [illegible]bove the head. I[illegible] the supine position the pedal axle of the bicycle was located 12—15 cm abov[illegible] the table and the stroke radius was 18 cm. During the exposures the arms were held [illegible]bove the head.

By means of pilot tests in both body positions [illegible] work load was selected which the subjects could sustain for [illegible]bout one hour and which increased their heart rates t[illegible] the levels found in the earlier circulatory investigations, *i.e.* after 10 mi[illegible] work 148 beats/mi[illegible] [illegible]th sitting and 128 beats/min in th[illegible] supine position (Ekelund and Holmgren 1964, Ekelund 1966). Resting values were obtained i[illegible] the same body position as during the subsequent work period. The exercise then began [illegible]t the selected load, which was held constant during the whole experiment, and the subjects exercised until exhaustion. Exposures were made after 4—6 and 15 mi[illegible] work and then [illegible]t different times depending on the heart rate. The final exposure were always made [illegible]bout one m[illegible]te before the subjects were unable to continue the experiment, *i*[illegible] almost exhausted.

Results

Material

The anthropometric data are presented in Table I.

The ECG at rest during and after work in the sitting position was normal in all [illegible] subjects. The subjects were of ordinary physical fitness with a mean rate of work [illegible]

TABLE I. Anthropometric data in ten healthy young men studied with heart volume determination during prolonged exercise. THb, g = total [illegible]mount of hemoglobin, W_{170}, kpm/min, = rat[illegible] of work that could be performed at [illegible] heart rate of 170 beats/min

Case no.	Age, years	Height, cm	Weight, kg	Heart vol. ml	THb, g	Blood vol., l	W_{170} kpm/min [illegible]
1	26	186	68	785	690	5.70	850
[illegible]	25	183	70	775	800	6.25	1000
3	24	188	72	700	815	6.15	1000
4	24	183	70	795	775	6.15	1150
5	21	189	78	770	900	7.05	1300
6	23	174	76	—	—	—	1050
7	23	186	69	—	—	—	1000
8	23	182	74	905	810	6.25	1200
9	27	177	63	803	705	5.50	1100
10	19	176	80	835	770	6.25	1050

of 1025 kpm/min (range 900—1300) at a heart rate of 170 beats/min. The relationships between heart volume and total amount of hemoglobin, heart volume and working capacity and working capacity and total amount of hemoglobin, were within the normal range of variation reported from this laboratory (Holmgren, Jonsson and Sjöstrand 1960).

Heart volume at rest in supine and sitting position

Exposures were made at rest both in sitting and supine position. These exposures were also used as a control of the triggering device response to different positions of the oesophagus electrode.

The exposures at rest were made at different intervals after the subjects had been placed to exercise position. The individual values are not given in the table but the tendency is the same as reported by Holmgren and Ovenfors (1960) with a smaller heart volume at rest sitting on the bicycle than in the supine position, mean difference 18.7 per cent ($P<0.01$).

In the sitting position heart volume increased on the transition from rest to work from an average at rest of 793.0 ml (n = 9 range 627—967) to an average of 825.4 ml (range 713—970) after 3—9 min exercise. In the supine position the resting heart volume averaged 974.7 ml (n=6, range 843—1130) and during the transition from rest to work there was a decrease of the same magnitude as the increase in the sitting position, to an average volume of 935.8 ml (range 850—1073) after 3—7 min work. Neither the difference in the sitting position nor the difference in the supine position was significant, $0.1<P<0.3$.

Heart volume during prolonged exercise in the sitting position

In the sitting position the work load was on an average 760.0 kpm/min (range 700—900) which was 70.9 per cent (range 62.5—80.0) of the rate of work performed at a heart rate of 170 beats/min (W_{170}) or 59.5 per cent (range 51.5—70.2) of the extrapolated maximal oxygen uptake (Åstrand 1960). The heart rate after a mean work time of 5.9 min (range 3—9) was 139.5 beats/min (range 128—154). The final heart rate was on an average 171.0 beats/min (range 150—182) after an average work time of 56.5 min (range 34—75.)

The values for heart volume during exercise in the sitting position are presented in Table II. During the prolonged continuous exercise there was a continuous decrease. The total decrease was on an average 81.1 ml (range 22—200) or 10.4 per cent (range 2.9—21.7) of the heart volume value obtained after 3—9 min exercise. This decrease was highly significant ($P<0.001$). One minute before exhaustion the heart volume increased in 6 of the 10 subjects by on an average, 52.5 ml (range 35—85), in spite of a further increase in heart rate from an average of 166.3 beats/min (range 146—176) to 170.0 beats/min (range 150—182). In two cases the heart volume remained unchanged while the heart rate increased from an average of 170.0 beats/min to 173.3 beats/min, and in one case it decreased by 50 ml with an increase in heart rate of 8 beats/min. The observed increase was, however

Table II. Heart rate (HR, beats/min) and heart volume (ml) at various times during prolonged exercise in the *sitting* position in ten healthy young men

Case no. Load, kpm/min	Time min	HR, beats/min	Heart volume, ml	Case no. Load, kpm/min	Time min	HR, beats/min	Heart volume ml
1	8	140	747	6	5	152	713
700	15	157	793	750	10	152	770
	30	175	725		23	160	725
	40	178	730		45	172	680
	45	180	728	50	50	174	680
					51	175	720
2	6	154	810				
800	15	169	743	7	8	128	800
	25	176	755	700	18	151	740
	30	176	710		25	157	763
	35	182	750		34	162	760
					45	168	680
3	9	148	780		60	171	670
700	17	158	750		65	174	668
	41	161	770				
	60	166	768	8	3	135	820
				750	6	148	777
4	6	143	920		19	160	750
800	15	147	897		61	164	750
	30	158	790		72	170	835
	40	154	810				
	52	159	830	9	3	146	775
	60	160	830	750	9	160	710
	70	162	770		20	166	705
	75	170	720		25	171	670
					34	174	705
5	6	156	843				
900	40	161	780	10	3	133	970
	50	167	730	750	9	142	930
	60	169	795		41	148	900
					60	146	900
					68	150	950

not significant ($P < 0.2$) which is partly due to the small number of observations. If the last determinations are excluded, there was a significant regression of the heart volume on heart rate with a negative slope and a correlation coefficient $r = -0.66$.

The equation for this regression

HV = 1363—3.75 HR, S.D. = ± 56, n = 48, $P < 0.001$

The corresponding equation for the six subjects which also took part in the examination in the supine position

HV = 1453—4.33 HR, r = —0.72, S.D. = ± 60, n = 23, $P < 0.001$

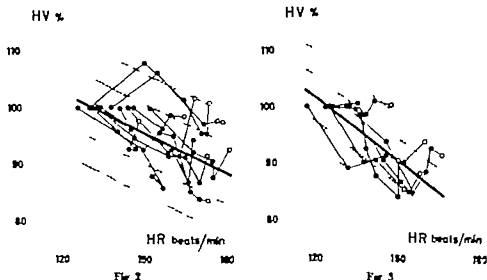


Fig 2 Heart volume, HV in per cent of the first exercise heart volume in relation [illegible] heart rate HR beats/min, during prolonged exercise [illegible] constant work load [illegible] the sitting position. Open circles represent determinations obtained close to exhaustion. Full line indicates the regression [illegible] individuals of all values except the final ones. Broken lines indicate S.D

Fig 3. Heart volume HV in per cent of the first exercise heart volume in relation to heart rate HR beats/min, during prolonged exercise [illegible] constant work load in the supine position. Symbols [illegible] in Fig. 2 Regression line for six individuals of all values except the final ones.

If the last heart rate values for each subject are entered into the first regression equation, one obtains a calculated heart volume which is almost significantly smaller than the measured heart volume the mean difference being 42.5 ml ($P <$ < 0.05)

To diminish the influence of body size a relative heart volume, HV_{rel}, was calculated. HV_{rel} is for each individual equal to the actual heart volume in per cent of the first heart volume determined during exercise

The equation for this regression

$HV_{rel} = 133 - 0.245$ HR, $r = -0.54$ S D $= \pm 5.0$, $n = 43$ P [illegible]

See Fig 2

Heart volume during exercise in the supine position

In the supine position the work load was on an average 758 kpm [illegible] [illegible] 00—900 which was 66.9 per cent range 63.6—69.2) of W [illegible] or 54.3 per cent range 50 [illegible] 58.3 of the extrapolated maxima [illegible] The initial heart rate after [illegible] min work time of 4.5 min (range 3—7 [illegible] min range 119—134 The final heart rate was on an average [illegible] beats/min range 149—167 after mean work time of 64.0 min (range 22 [illegible] [illegible] no 5, worked for 38 min but [illegible] [illegible] responses were obtained [illegible] 22 min

TABLE II Heart rate, HR, beats/min, and heart volume ml, at various times during prolonged exercise in the *sitting* position in ten healthy young men

Case no. Load, kpm/min	Time, min	HR, beats/min	Heart volume, ml
1	8	140	747
700	15	157	793
	30	173	723
	40	178	730
	45	180	728
2	6	154	810
800	15	169	45
	25	176	735
	30	176	710
	35	182	750
3	9	148	780
700	17	158	750
	41	161	770
	60	166	768
4	6	143	920
800	15	147	897
	30	158	790
	40	154	810
	52	159	850
	60	160	830
	0	162	770
	75	170	720
5	6	156	843
900	40	161	780
	50	167	730
	60	169	795
6	5	152	715
750	10	152	770
	25	166	723
	45	172	680
50	50	174	680
	51	175	720
7	8	128	800
700	18	151	740
	25	157	763
	34	162	760
	45	168	680
	60	171	670
	65	174	668
8	3	135	820
750	6	148	777
	19	160	750
	61	164	750
	77	170	833
9	3	146	773
750	9	160	710
	20	166	705
	25	171	670
	34	174	705
10	5	133	970
750	9	142	930
	41	148	900
	60	146	900
	68	150	950

not significant ($P<0.2$) which is partly due to the small number of observations. If the last determinations are excluded, there was a significant regression of the heart volume on heart rate with a negative slope and a correlation coefficient $r = -0.66$.

The equation for this regression

HV = 1563 – 3.75 HR, S.D. = ± 56, n = 43, $P < 0.001$

The corresponding equation *for the six subjects which also took part* in the examination in the supine position

HV = 1453 – 4.33 HR, $r = -0.72$, S.D. = ± 60, n = 25, $P < 0.001$

at the end of work by on an average, 42.0 ml (range 30—63) and the heart rate increased at the same time from 133 0 beats/min (range 131—135) to 157 0 beats/min (range 151—161) In three cases the heart volume decreased further on an average 22.7 ml (range 10—30) at the end of work with an increase in heart rate from 149 7 to 156 3 Neither change was, however significant.

If the last determinations are excluded there was a significant regression of the heart volume on the heart rate with a correlation coefficient $r = -0.57$ The equation for this regression $HV = 1709 - 5.83\ HR$, $S.D = \pm 94$ $n = 27$ $P < 0.005$

To diminish the influence of body size a relative heart volume, HV_{rel}, was calculated. HV_{rel} is for each individual equal to the actual heart volume in per cent of the first heart volume determined during exercise. If the relative heart volume value is used, the correlation coefficient is higher $r = -0.71$ The equation for this regression

$HV_{rel} = 148 - 0.383\ HR$, $S.D = \pm 4.2$ $n = 27$ $P < 0.001$

See Fig 3

If the last heart rate values in each subject are entered into the regression equation for the relative heart volume, the calculated relative heart volumes are 3.5 per cent smaller than the measured volumes, but the difference is not significant ($0.1 < P < 0.2$)

The regression line for HV_{rel} on heart rate has a significantly higher level in the supine position than in the sitting ($P < 0.001$) but the slope is not significantly different.

Discussion

The subjects used in this investigation were of ordinary physical fitness and had about the same anthropometric data the subjects used in earlier investigations of the effect of prolonged exercise on the circulatory and respiratory adaptation (Ekelund and Holmgren 1964 1965 Ekelund 1966, 1967) It seems probable, therefore that they should be comparable with the previous subjects as regard the circulatory and respiratory responses to long-term non-steady stat exercise within the range of working intensities employed.

The present method of determination of the heart volume during exercise has a reproducibility of the same order a reported for determinations at rest (Holmgren and Ovenfors 1960 Hellström and Holmgren 1966) Thus the 95 per cent confidence level of a single determination is of the order of ten per cent

The calculation of the heart volume employs the three diameters of an ellipsoid of revolution The validity of a comparison of two measurements of heart volume under different experimental conditions, depends upon whether these diameters all change to the same relative extent. In the present investigation the inter-relationship between the three diameters did not change significantly

To get an absolute value for the heart volume one must take into consideration the influence of different heart configurations, as was done when calculating the heart

volume in the prone position according to Larsson and Kjellberg (1948). This has not been done in the present investigation when measuring during exercise. The absolute values for heart volume in th prone position can therefore not be compared with the corresponding values in the supine position.

The aim of the present investigation vas to measure the total heart volume at the end of diastole, just before the beginning of the isometric contraction. As is illustrated in Fig 1 this was attained even at heart rates up to 182 beat /min.

The value for the total heart volume obtained in this way is expected to represent the ventricular volume to a major extent since both atria should be maximally contracted. The observed changes n total heart olume should consequently reflect changes in the end-diastolic olume to a major extent. It is, however important to consider that the changes in total heart volume during the heart cycle are included in the ariation of 4 per cent of a single non-triggered determination (Hellströen and Holmgren 196F

Left ventricular volume has been estimated by a number of methods, the validity of which are still under discussion (Soloff 1966 The results observed during exercise in normal subjects are not consistent. Paley and Leonard (1965) and Gorlin et al. (1965) both using the thermodilution technique, found no change in left ventricular end-diastolic volume during exercise lasting 4—10 min. Stroke olume increased during th first 4—10 min of exercise but as a result of a more complete emptying of the ventricle Braunwald *et al* 1963 made a cineradiographic analysis of the ventricular dimensions from the movement of silver clips sewn to the entricular wall in nine patients with minor heart disease These investigators found a decrease in both right and left entricular dimensions during min exercise of low intensity It is, however difficult to convert their observations of linear changes in one plane to volume changes in normal subjects during heavy exercise

The present observations of a continuous decrease of the end-diastolic heart olume with increasing heart rate during non-steady state exercise, both in the upine and in the sitting position, agree well with the reports of earlier tudies on stroke volume in the sam experimental situation Ekelund and Holmgren 1964 Ekelund 1966

The explanation f both changes ma be an increase of blood in the capacity vessels a shift in blood which increases with time and is probabl a esult of a successive slackening of the mpathetic tone of these essels or a diminished response in the ascular wall to an unchanged or increased stimulus

It is interesting to observe the small influence of body position on direction and magnitude of the changes in heart olume and stroke olume during prolonged exercise. The explanation is probably to be found in the higher level of mpathetic tone at which the capacity essels operate in upright position Vendsalu 1960 Body position is thus not of major importance for the magnitude of the hanges in the present material.

Two types of reactions of the heart olume were observed close t exhaustion. In all subjects there was an increase in heart rate between the two last measurements. Heart olume either continued to decrease or increased almost significantly in 6 out

of 10 subjects in the sitting position and in 3 out of 6 in the supine. Since no information of cardiac output and stroke volume at that very moment is available it is difficult to evaluate this observation. No increase in central venous pressure at the end of half an hour's heavy work at a heart rate of 170 beats/min was observed in well trained subjects (Holmgren 1956) or at exhaustion after 1 hour's exercise in ordinary physical fit subjects (Ekelund and Holmgren 1964)

The increase in heart volume may thus be explained by a shift of blood within the capacity vessels due to an increase in the sympathetic tone caused by exhaustion or a change in the elastic and resistive properties of the myocardium so that the heart is dilated in spite of the filling energy remaining constant. If stroke volume should remain constant or decrease, this implies that the function of the myocardium has been changed.

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volume in the prone position according to Larsson and Kjellberg (1948). This has not been done in the present investigation when measuring during exercise. The absolute values for heart volume in the prone position can therefore not be compared with the corresponding values in the supine position.

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The present observations of a continuous decrease of the end-diastolic heart volume with increasing heart rate during non-steady state exercise, both in the supine and in the sitting position, agree well with the reports of earlier studies on stroke volume in the same experimental situation (Ekelund and Holmgren 1964, Ekelund 1966).

The explanation of both changes may be an increase of blood in the capacity vessels, a shift in blood which increases with time and is probably a result of a successive slackening of the sympathetic tone of these vessels or a diminished response in the vascular wall to an unchanged or increased stimulus.

It is interesting to observe the small influence of body position on direction and magnitude of the changes in heart volume and stroke volume during prolonged exercise. The explanation is probably to be found in the higher level of sympathetic tone at which the capacity vessels operate in upright position (Vendsalu 1960). Body position is thus not of major importance for the magnitude of the changes in the present material.

Two types of reactions of the heart volume were observed close to exhaustion. In all subjects there was an increase in heart rate between the two last measurements. Heart volume either continued to decrease or increased almost significantly in 6 out

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Local Myocardial Blood Flow Measured by Hydrogen Polarography; Distribution and Effect of Hypoxia

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Received 13 December 1966

Abstract

AUKLAND, K., F. KIIL, J. KJEKSHUS and G. SEMB. *Local myocardial blood flow measured by hydrogen polarography; distribution and effect of hypoxia.* Acta physiol. scand. 1967. 70. 99—111.

Local myocardial blood flow was measured in open chest dogs by polarographic recording of hydrogen desaturation with needleshaped platinum electrodes. A potential of + 0.3 V relative to KCl saturated calomel electrode was applied to the platinum electrode to minimize oxygen sensitivity. The influence of convection in the medium on electrode current was investigated in vivo. Myocardial hydrogen desaturation recorded with tissue electrodes gave acceptable monoexponential curves in about 60 per cent of all electrode sites. Possible mechanisms of multi-exponential curves which were discarded are discussed. Good agreement was obtained between two electrodes in one ventricle and between left ventricular and coronary sinus recordings, and arguments are given that hydrogen clearance equals blood flow in the range of 50—450 ml/min/100 g. Myocardial blood flow was on the average 11 per cent lower in the right than in the left ventricle (anterior walls) and this relationship was unchanged during dextran infusion and spontaneous variations of myocardial flow. Right/left flow ratio also remained unchanged when myocardial flow was increased up to four times by hypoxia. The flow rise was such as to maintain or even increase the amount of oxygen delivered to the myocardium of both ventricles.

The determinants of myocardial blood flow are only partially known. Factors such as perfusion pressure, intra myocardial pressure, work load, heart rate, nervous stimuli, hypoxia and other metabolic stimuli have all been shown or suspected to be of importance (Berne 1964). Since ventricular pressure and work load may be varied independently for each ventricle, measurement of local myocardial blood flow in the left and right ventricle might serve to clarify the importance of these parameters. This approach requires a method for simultaneous and repeatable measurements of blood flow in the two ventricles. Direct methods, such as measurement of blood flow in the left and right coronary artery, coronary sinus, etc. can only give approximate values for local perfusion of the myocardium of the left and right ventricle.

require surgical procedures which may reduce the physiological significance of the results.

The distribution of myocardial blood flow at a given moment can be estimated from local Rb [86] uptake (Love and Burch 1957 Love and O'Meallie 1963) *However* the value of this method for studying myocardial hemodynamics is limited by the fact that only one measurement can be obtained in each animal, which cannot therefore be used as its own control.

Regional or local myocardial flow can also be estimated by recording wash-out of Krypton-85 or Xe 133 injected into a coronary artery or into the myocardium (Herd *et al* 1962 Linder 1966) but neither of these techniques would seem to permit simultaneous and repeated measurements in two regions. In the present study we have used polarographic recording of local hydrogen gas desaturation by platinum electrodes, which has been shown to give a good estimate of local myocardial blood flow in the normal range (Aukland Bower and Berliner 1964 Neely *et al.* 1965) The present paper describes further methodological improvements. We have also examined the reliability of the method over a wider flow range, and to what extent measurements with a single electrode are representative for a whole ventricle. Furthermore, as a basis for studies on the effect of selective changes in left and right ventricular pressure and for studies on coronary artery occlusion the distribution and spontaneous variations of left and right myocardial blood flow and the effect of arterial hypoxemia will be described.

Methods

Polarographic measurement of hydrogen tension

The electronic setup used for altern ting readings of three electrode currents is show in Fig. 1 The resistance in the electrode circuit was 10^{6} or 5 10^{5} Ohm. A Keithley Instruments Microvolt Ammeter 150A, was used on the millivolt setting The input resistance in this range is 9 10^{7} Ohm, nd witching from one platinum electrode to another will not appreciably infl ence the effective electrode poten ial The output signal was recorded on Honeywell-Brown Electronic Recorder. It has previously been shown that the current produced by hydrogen oxidation t he platinum electrode polarized at the potential of KCl saturated calomel electrode is linearly related to hydrogen ension, and that he relative ffect of oxygen reduction this potential is 10—30 per cen (Aukland *et al* 1964) Oxygen reduction was therefore studied *in vitro* at arying polarizing potential and it was found tha th unwanted oxygen ffect ca be greatly reduced by applying

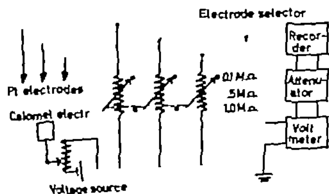


Fig 1 Schematic wiring diagram for polarographic determina ion of hydrogen.

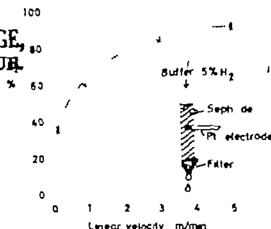


Fig. 2. Influence of convection on hydrogen oxidation current (in) t constant H concentration measured with two lec trodes (circles and crosses) I , in per cent of *maximum current*, obtained t linear elocity of bout 20 cm/min. Inset indicates experimental setup.

posith potential was the KCl saturated calomel electrode I the pO_2 range of 0—150 mm Hg the following relative effect of changes in pO compared to identical changes in pH were found at the polarizing potential of + 0 1 V 5 at + 0.2 V 2 , and at + 0 3 V less than 1 ,o Since hydrogen current is rather constant in the potential range of 0— + 0.3 V the latter polarization potential was used in the present study The ffect of hanging pH by 0.1 unit I the range of pH 7—8 as found to be negligible also with polarizing potential of + 0.3 V

The influence of convection in the medi m on electrode current was also tested in vitro. T old grom turbulence around be lectrode, and some extent simulate capillary flow the electrode was placed in Sephadex column, as shown in Fig 3. Phosphate buffer pH 7 4, equilibrated with 5 hydrogen gas in air was allowed t flow through the column various elocities. As evident from Fig 3 the curren measured from tw different electrodes was considerabl nfluenced by flow rate, especially in he lower range. (With zero convection steady state current can be obtained, since the current will continue to fall with increasing hydrogen depleted olume around the lectrode (Kolthoff and Lingane 1852))

Experimental procedure

Acute experiments ere performed on mongrel dogs, weighing 15— 1 kg Anesthesia was induced by sodium pentobarbital, 25 mg/kg and maintained by subsequen doses of 25—75 mg The thorax was opened by sternum-split procedure. Respiration was maintained by positi pressure AGA respirator The anterior portion of the pericardium was excised to giv broad access to the heart, which was suspended I the remaining pericardium. Care was taken to avoid stenosis of the large ems by traction A polyethylene ca heter was introduced into the aorta ia the righ carotid artery for arterial blood samples nd pressure recording. Another catheter as introduced into the left trium or en ricle via the auricular appendix for injection of hydrogen saturated saline and pressure recording Phasic nd erage pressures were recorded with Statham's pressure transducers and Sanborn mplifiers and recorder Arterial blood samples were draw anaerobically during each flow measurement Hemoglobin oxygen saturation was determined by the method of Refsum and Sveinsson (1956 and hemoglobin was estimated spectrophotometrically as cyanmethemoglobin Oxygen ontent as *calculated by the conversion factor of 1.36 ml O_2/g hemoglobin.* Blood pH as measured anaerobically ith Radiometer pH meter

The tissue electrodes ere made principally as previously described (Aukland *et al.* 1964) with he exception tha be shaft was made L-shaped so tha the extramural part of the electrode would rest on be heart surface The activ electrode p was 1— mm long Tw or more electrodes were *placed the myocardium of be left and right entricle about half-w y between pex and the* entricular base (areas schema ically indicated in Fig 4 inset) and tentatively into the middle layer of he myocardium Stitching the electrodes to the epicardium was ried but seemed to be of doubtful alue in be exposed heart The electrode positions were checked at the end of each experimen I three experiments platinum electrode mounted on heart ca heter was inserted nto he coronary sinus ia the right uricular appendix The design of the electrode tip as previously described (Aukland *et al.* 1964)

H drogen as added to the tracheal tube gi ing concen ra ion of bout 5 until myocardial hydrogen concentration as reasonably stable (he oltage drop in he electrode circuit was alw ys kept below 0 1 V) T provide rapid arterial desaturation hydrogen respiration was hen stopped

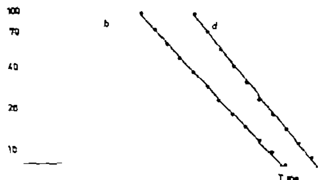


Fig 3 Evaluation of nonlinear desaturation curves. Curves and b discarded. Curves and d considered acceptable. Flow calculated from slope of unbroken line.

and manual injection of 50 ml 100 % hydrogen saturated saline int the left heart started at rate adjusted t maintai constant myocardial electrode current for another 20—60 seconds. When the intraventricular nfusion was cutely stopped, the desaturation of the myocardium was followed by alternate recording from two or three lectrodes.

Hypoxemia was produced by feeding the respirator with various oxygen-nitrogen mixtures. After cha ging gas mixture, an equilibration period of 5—10 minutes was allowed before flow measurement. Each hypoxemia period was preceded and followed by control period with air respiration.

Blood flow in ml/min 100 g tissue was calculated from the slope of the desaturation curves plotted on semilogarithmic paper according t the formula $F/W = 69.3/T_{1/2}$, where T is the half-time in minutes. The tissue/blood partition coefficient (1) and specific gravity of th myocardium assumed t be 1 00. Curves deviating considerably from linearity as exemplified in Fig 3, and b, were excluded for reasons giver in Discussion. Curves with slight deviation from linearity ere ccepted, nd flow was calculated from the average slope in the rang of 100—10 % desaturation. (Fig 3, and d)

Results

General description of the desaturation curves

Typical desaturation curves obtained simultaneously from three electrodes are shown in Fig 4 The intermittent recordings from each electrode were connected, smoothing out obvious artifacts, and recorded current minus rest current was plotted on a logarithmic scale against time, as shown in th inset. Although the slow components of the electrocardiogram were superimposed, the reading usually presented no major problems, and independent plotting and flow calculation by two persons generally agreed within 5 About one fifth of all electrode sites were discarded because of multi-exponential desaturation curves (cf Fig 3) However such deviations from linearity in the semi log plot remained fairly constant for a given electrode site throughout experiments of several hours duration, and t was obvious that reasonably good estimates of relative changes in blood flow could have been btained also from the discarded curves. When two satisfactory curves were obtained in one ventricle, the mean of their respective flow values were used in comparing l ft and right ventricular flow

Distribution of myocardial blood flow

Since the present technique allows simultaneous flow measurement with only three different electrodes, no attempt was made to map out the flow distribution of the entire myocardium. It was of importance to examine however whether flow values

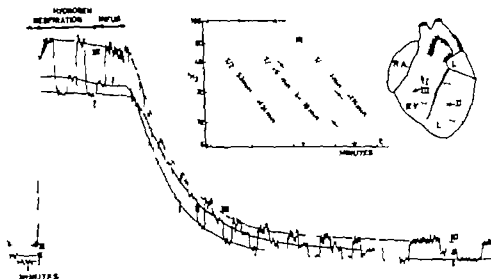


Fig. 4 Original desaturation curves recorded simultaneously from two electrodes in right ventricle (I and III) and one electrode in left ventricle (II) Inset shows semilogarithmic plot of desaturation curves and electrode positions.

obtained from one electrode site may be considered representative for one ventricle, and, furthermore, if the myocardial blood flow is different in the right and left ventricle.

Comparison of two electrode sites in the same ventricle

Simultaneous measurements with two electrodes in the same ventricle were made with 15 electrode pairs in 13 experiments. Seven pairs were located in the left ventricle and eight in the right ventricle within the areas described above and schematically indicated in Fig 4 The distance between the electrodes varied between 10 and 40 mm. An average of 3 8 flow measurements were made under control conditions with each pair The average difference for each pair was calculated in per cent of their mean flow giving an overall average difference of 12 6 (left 13 6 and right 11 8) with the following distribution 0—10 % difference 8 pairs, 10—20 4 pairs, and 20—35 3 pairs. The "standard error of the method assuming completely homogeneous perfusion in each ventricle, was 11 9 % (Calculated as the square root of the sum of the squared difference in each pair in per cent of their mean, divided by twice the number of pairs) Within the areas investigated (Fig 4 inset) no consistent regional differences were observed

Distribution of blood flow between left and right ventricle

Simultaneous recording in left and right ventricle were made in 20 experiment including 108 flow measurements during control conditions. The average left ventricular blood flow in this series was 99 7 ml/min 100 g (SE 9 1 n = 20 and

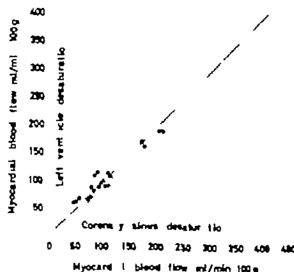


Fig. 5. Relationship between myocardial flow calculated from simultaneous left ventricular and coronary sinus desaturation curves. Four experiments indicated by different symbols.

right ventricular flow was 87.9 ml/min 100 g (SE 8.7 n = 20). In individual experiments the ratio between right and left myocardial blood flow varied between 0.57 and 1.37 with an average of 0.89 (SE 0.047 n = 20). Comparing simultaneous measurements, the blood flow per gram myocardium in the left ventricle is thus significantly higher than in the right.

Comparison of desaturation curves from left ventricle and coronary sinus

Simultaneous recordings of hydrogen desaturation in the coronary sinus and the left ventricle were performed with four tissue electrodes in three experiments, including a total of 30 desaturation runs. The coronary sinus desaturation curves sometimes showed a slight deviation from the ideal exponential curve below the 90 desaturation level suggesting a small slower flow component in the drainage area for the coronary sinus. Rapid initial desaturation suggestive of arterio-venous shunts was not recorded from the coronary sinus in any experiment. For comparison with left ventricular flow the practically linear slope down to 90 desaturation was used for flow calculation. High myocardial flow was induced by hypoxia. As shown in Fig 5, a fairly constant relationship was found between left ventricular flow from each electrode site and flow calculated from coronary sinus desaturation over a wide flow range.

Spontaneous variations and effect of dextran (Macrodex) infusion

In most experiments the myocardial flow in control periods remained fairly constant for several hours. In some instances a gradual decline or more rarely an increase in flow was observed, and then in the same direction for two electrode sites in the same ventricle as well as in the two ventricles. The changes were usually proportional, although some variations in both intra and interventricular ratios were observed occasionally.

TABLE I Effect of Macrodex Infusion on Myocardial Blood Flow in Right and Left Ventricle

Time	Heart rate	Mean pressure		Myocardial blood flow	
		Aortic	L. ventr	Left	Right
ml		mm Hg	mm Hg	ml/min 100 g	ml/min 100 g
0	145	55	35	57	61
50	150	55	30	60	68
58—75	Macrodex 6	200 ml L			
70	165	85	50	132	151
105	170	85	50	159	159
135	160	85	45	116	114

In a few experiments an intravenous infusion of 100—300 ml Macrodex was given because of a low arterial pressure at the end of the surgical procedure. This produced an increase in myocardial blood flow in both ventricles, the right/left ratio remaining unchanged (Table 1)

Effect of hypoxia on local myocardial blood flow

The effect of hypoxia on left and right myocardial blood flow was studied in seven experiments, including 14 hypoxia periods with control periods before and after In control periods the average arterial hemoglobin oxygen saturation was 92 and oxygen content 13 1 vol. The average blood pH was 7 41 and remained unchanged throughout each experiment including hypoxia periods. In hypoxia periods arterial hemoglobin saturation was reduced to levels between 79 and 20 The myocardial blood flow in control periods averaged 99 1 ml/min 100 g in the left ventricle and 89 1 ml/min 100 g on the right side A significant rise in blood flow was observed

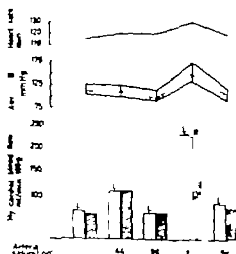


Fig Effect of hypoxia on left L and right R myocardial blood flow recorded with one electrode in each ventricle Hgb 12 – 14 0 g 100ml

TABLE II. Effect of Hypoxia on Myocardial Blood Flow in Right and Left Ventricle

Time	Hb	Art. O_2-sat.	Arterial O_2-cont	Heart rate	Mean pressure		Myocardial blood flow		Flow ratio Right/Left
					Aortic	L ventr	Right	Left	
min	g/100 ml	%	ml/100 ml		mm Hg	mm Hg	ml/min 100 g	ml/min 100 g	
0	11.8	92	14.8	160	110	55	65	88	74
11	11.9	58	9.4	165	125	65	134	177	76
30	11.5	89	13.0	160	115	65	67	90	75
44	11.8	54	9.0	170	125	65	127	173	73
59	10.9	94	13.9	160	115	55	69	90	77
79	12.6	16	2.7	120	145	65	308	445	69
92	10.5	95	13.5	165	120	60	64	86	74

in all hypoxia periods in both ventricles, with return close to control values upon changing to air breathing (Fig. 6). The flow increment was usually proportional to control flow, as evident from Table II showing an experiment with considerably higher flow in the left than in the right ventricle. This is also obvious from Fig. 7 which gives the pooled data from all hypoxia experiments with an average right/left flow ratio of 0.87 during hypoxia compared to the average control ratio of 0.88.

Moderate hypoxia usually produced a small rise in systolic pressure in aorta, whereas diastolic and mean aortic pressures as well as heart rate showed no or inconsistent changes. More pronounced hypoxia, below 50 % saturation, produced a marked rise in systolic and diastolic pressures (mean aortic pressure increment up to 50 mm Hg), whereas heart rate showed inconsistent changes. Myocardial blood flow was poorly correlated to these parameters, as evident from Table II.

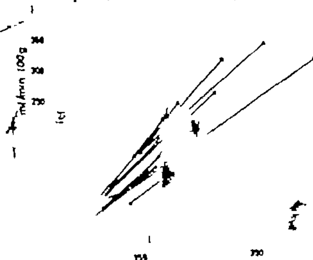


Fig. 7. Effect of hypoxia on right and left myocardial blood flow in 7 dogs (indicated by different symbols). Control flow during air breathing (in the left) represents the average of pre- and post-hypoxia measurements. Average control right/left ratio for all animals, 0.88, indicated by dotted line.

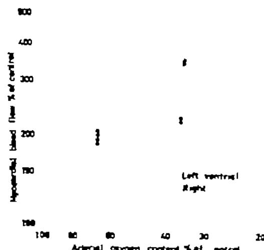


Fig 8. Increase in myocardial flow related to arterial oxygen content. Logarithmic scale on abscissa and ordinate. Diagonal line indicates unchanged arterial oxygen supply. Average results obtained in intact animals by Hackel *et al.* (1954, 1956) indicated by triangle and squares respectively

The percentage increase in myocardial blood flow in all experiments showed positive correlation to the lowering of arterial oxygen content. This is evident from Fig 8 where myocardial blood flow and arterial oxygen content are presented in per cent of control values. The diagonal line indicates the flow increase necessary to maintain unchanged oxygen supply (arterial oxygen content × flow) to the myocardium. It will be seen that the oxygen supply usually remained constant or even increased during hypoxia under the conditions of the present experiments. For comparison, literature data (Hackel *et al.* 1954 1956) for the intact heart measured with coronary sinus catheterization are also included in the diagram

Discussion

The feasibility of measuring local myocardial blood flow in the contracting bypassed dog heart by polarographic recording of hydrogen gas desaturation has been demonstrated previously. Comparison between local myocardial blood flow in the left ventricle and directly measured coronary venous outflow showed good agreement in the normal flow range (Aukland *et al.* 1964). In the present study the possibility of measuring myocardial flow in both ventricles over a wider flow range and over periods of several hours was examined in more detail. The technique was modified by using a polarizing potential of − 0.3 V

The insertion of the electrode may disturb local circulation and produce a layer of uncirculated tissue blood or tissue fluid around the electrode. Such a layer reduces electrode current and also produces an initial delay in the desaturation curve but does not influence the slope of the curve (Aukland 1965). This conclusion is experimentally supported by the present observation of good agreement between left ventricular and coronary sinus desaturation curves, even at very high myocardial blood flow. This finding also indicates that correct absolute flow values are o

in the left ventricle since venous desaturation curves have shown good agreement with independent flow measurements in the flow range of 40—90 ml/min 100 g in the myocardium and up to 500 ml/min 100 g in the kidney (Aukland *et al.* 1964). Neely *et al.* (1965) using a polarizing potential of about 0.2 V also reported good agreement with directly measured coronary venous outflow in the normal flow range. It is obvious that sudden arterial desaturation becomes more important with increasing blood flow. The discrepancy between renal venous outflow and venous desaturation rate observed at high flow by Neely *et al.* (1965) is probably due to too slow arterial desaturation, since hydrogen was given only by respiration in their experiments.

The reliability of the method is also corroborated by the fairly good agreement between two electrodes in the same ventricle, giving a standard error of the method of 11.9 %. This variability obviously includes both methodological errors and real flow differences between various areas in the same ventricle. Although the myocardium seems to have a remarkably homogeneous flow, local or regional differences are nevertheless likely, as also indicated by rubidium clearance studies (Chansky and Levy 1962). Occasional differences as high as 30 per cent between two neighbouring electrodes show, however, that measurement with a single electrode is not sufficient to give an accurate value for average blood flow in a whole ventricle. On the other hand, the proportional variations in flow values from different electrodes indicate that the direction and percentage changes in blood flow are reliably reflected by a single electrode.

In control periods local flow in the left ventricle was about 12 higher than in the right, the difference being significant at the 5 level. A somewhat larger difference has been observed with Rb^{86} uptake studies (Love and Burch 1957, Chansky and Levy 1962). On the other hand, MacLean *et al.* (1961) found higher uptake of intra-arterial injected microspheres in the right than in the left ventricle. The reason for this discrepancy is unclear.

It is well established that hypoxia increases myocardial blood flow mainly by reducing coronary vascular resistance (Berne 1964), as evident also from the present study. The observed increase in blood flow during hypoxia was generally such as to maintain or even increase the amount of oxygen delivered by arterial blood both to the left and the right ventricle. This is in agreement with results obtained in closed chest dogs with coronary sinus catheterization (Hackel *et al.* 1954, 1956) as shown in Fig. 8. Much smaller effect of hypoxia on coronary blood flow with reduction in oxygen delivery has been observed in perfused hearts by Berne, Blackmon and Gardner (1957). In their experiments, the myocardial oxygen requirement was met mainly by increased oxygen extraction which in their preparation was low in control periods.

In contrast to the present findings, Love and Tyler (1965) found that hypoxemia produces a proportionately greater fall in myocardial vascular resistance in the right than in the left ventricle, as calculated from Rb^{86} uptake. However, they do not seem to have taken into account the fall in Rb-extraction with rising flow (Love and

O Mealbe 1963) which might contribute to the observed difference because of the higher uptake in the left ventricle in control animals.

The justification for rejecting multi-exponential desaturation curves and the procedure for flow calculation from curves deviating slightly from linearity in semi log plots is discussed in the following Non-linear curves might be due to 1) uneven capillary blood flow in the region of the electrode, 2) rapid desaturation of tissue urrounding small arteries, or 3) diffusion of gas through the surface of the heart

1) The single exponential hydrogen desaturation curves obtained in the coronary sinus and also the exponential average tissue desaturation observed with Kr* (Herd *et al.* 1962) suggest a homogeneous perfusion of the myocardium. However consider able deviations from homogeneity such as suggested by Rb* uptake studies (Love and Burch 1957) might not be detected by these methods To a considerable ex ent such variations should be averaged by using relatively large electrodes, 1—? mm long, as in the present studies. Such electrodes therefore seem preferable to micro-electrodes, which are more likely to be influenced by variations in flow in smaller tissue elements.

2) An experimental evaluation of the effect of small arteries seems unfeasible, nd we have therefore estimated, theoretically the desaturation rates at various distances from a small artery It was assumed that hydrogen concentration within the artery is suddenly lowered to, and maintained at zero and that hydrogen is removed from the surrounding tissue only by the artery A tissue diffusion coefficient for hydrogen of 5 10⁻⁴ cm sec⁻ was estimated from measured diffusion coefficients for oxygen and carbon dioxide in aorta and other tissues (Kirk and Laursen 1955, Thews 1960) assuming the diffusion coefficient to be inversely proportional to the square of mole cular weight. Fig 9 shows desaturation curves calculated by Crank Nicholson methods (Crank 1956) on a digital computer at various distances from center of an artery wi h internal diameter 100 μ The diagram indicates that such an artery may

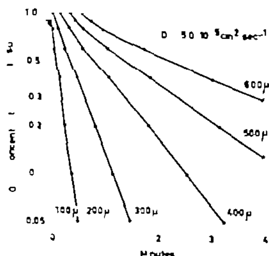


Fig 9 Calcula ed desaturation curves at arious distances from he enter of artery ith radius 50 μ Assumptions complet mutual equilibration and instantaneous desaturation of arterial blood erro time D 5 10 cm sec

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1) The single exponential hydrogen desaturation curves obtained in the coronary sinus and also the exponential a erage tissue desaturation observed with Kr^{85} (Herd *et al* 1962) suggest a homogeneous perfusion of the myocardium. H wever consider able deviations from homogeneity such as suggested by Rb^{86} uptake studies (Love and Burch 1957) might not be detected by these methods. To a considerable extent such variations should be averaged by using relatively large electrodes, 1—2 mm long, as in the present studies. Such electrodes therefore seem preferable to micro-electrodes, which are more likely to be influenced by variations in flow in smaller tissue elements.

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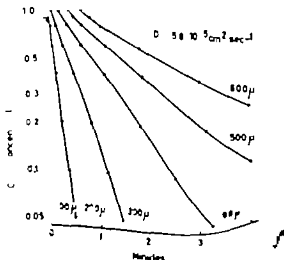


Fig. 9 Calculated desa ration urves arious distances from he center of artery with radius 50 Assumptions complete initial equilibration nd instar taneous desaturation of arteri l blood zero time. D = 5 10 cm sec

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Effect of the Supply of Oxygen on the Tensile Strength of Healing Skin Wound and Granulation Tissue

By

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Received 9 January 1967

Abstract

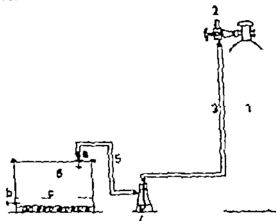
KULONEN, E., J. NIINIKOSKI and R. PENTTINEN. *Effect of the supply of oxygen on the tensile strength of healing skin wound and granulation tissue.* Acta physiol. scand. 1967. 70. 112—115.

Experimental granulomas were induced in rats with subcutaneously implanted viscose cellulose sponges. The rats were kept for 10 days in normobaric atmospheres containing 21, 35 or 70 (v/v) oxygen. After killing, the tensile strengths of the granulomas and skin wounds were measured. In granulomas the tensile strengths were: in 21 % O 107.2 ± 9.7 g (25 determinations), in 35 % O 190.2 ± 18.5 g (41), in 70 % O 221.0 ± 24.6 g (26) and in skin wounds 431.3 ± 32.8 g (25), 534.0 ± 22.8 g (40) and 574.1 ± 32.9 g (24) respectively. The analysis of variances confirmed that the variation between the groups is larger than within the groups (for the granulomas $P < 0.001$ and for the skin wounds $P < 0.005$).

This work was inspired by the finding that oxygen is necessary for the synthesis of collagen in incubated slices of sponge-induced granulation tissue (Kao et al. 1963). We wished to learn whether this dependence could be extended to whole animals living in normobaric atmosphere where the concentration of oxygen could be varied. A preliminary report has appeared (Niinikoski, Penttinen and Kulonen 1966).

Relevant observations in the literature are few. In rats which had been kept intermittently in hyperbaric (2 atm) oxygen the wound healing was retarded (Lundgren and Sandberg 1965). On the other hand, the regeneration of wound epithelium was more rapid at sea level than in the mountains of 3 000 m (Utkina 1958). Also, Beckham and Hitchcock (1965) reported that hyperbaric oxygenation of tissues may influence beneficially the healing of wounds. Intra-arterial injection of hydrogen peroxide improved the healing of leg sores (Balla et al. 1964). They observed that no systematic study has been performed on the effect of oxygen on the wound healing with reference to the tensile strength.

Fig. 1 Experimental arrangement From a gas cylinder (1) the oxygen-nitrogen mixture flows (usually at the rate of 3 l/hr) through the reducing valve (2) and 5-mm tubing (3) to a washing bottle (4) which serves as both flow-meter and humidifier and then through further tubing (3) to box (6), where the rats are kept. The air-tight box (20 30×38 cm) is made of Perspex-sheets. There are small holes for the entry ( ) and the exit (b) of the gas. The rats lie on removable wire floor ( ) of stainless steel and the bottom of the box is covered with saw dust, which is changed every 3rd day



Experimental

Induction of granulomas. Two or four pieces of viscose cellulose-sponge ($20 \times 10 \times 10$ mm each) were implanted subcutaneously in the backs of male rats (Wistar strain, weight 200 ± 30 g). The pieces had been cut into two equal halves, fixed to the original position with stitches, and sterilized by boiling in 0.9 (w/v) NaCl solution for 30 min. The length of the skin wound was 3 cm and it was closed with five sutures of cotton yarn.

Arrangement of the variations in the supply of oxygen The set-up is shown schematically in Fig. 1 Three rats were kept simultaneously in each box (6) fed with the standard laboratory diet and water *ad libitum*. For the feeding and cleaning the boxes were opened once every day The cylinders (1) contained mixtures of oxygen and nitrogen (O 21 50 % and 100 (v respectively) I the boxes the final concentrations of oxygen were 18 35 and 70 respectively as ascertained with Scholander's apparatus (kindly placed to our disposal by the Department of Physiology University of Turku) The CO_2-concentration inside the boxes reached during the 24-hr period level of about 5—6 which caused some hyperventilation The relative humidity in the boxes was about 90 and the temperature 2—3 C above the ambient temperature (cf Halpern and Cole 1944 No signs of O_2-intoxication, *e. g* convulsions and pulmonary edema, were observed, probably because even the highest actual concentration of oxygen was no more than 70 %.

Measurements Ten days after the implantation the rats were killed and the tensile strengths of the granulomas and of the skin wounds were measured as described by Viljanto (1964) The granulomas were stored frozen for chemical analyses

Results and discussion

The averages of the tensile strengths are shown in Fig 2. The favorable effect of the increased oxygen concentration is clear especially in the range of 21—35 % O At higher concentrations the curves level off On the hyperbaric conditions we do not have any *experience* At lower concentrations of oxygen (about 12 %) the tensile strengths are decisively decreased in comparison with the results in air (336.2 ± 25.6 g for skin wounds and 43.8 ± 3.9 g for granulomas, n = 25 for both)

The most marked effect of oxygen in the tensile strength of the healing wounds is thus observed when its supply is varied starting from the composition of air This is not necessarily true for the epithelial regeneration where hyperbaric oxygen may be more efficient.

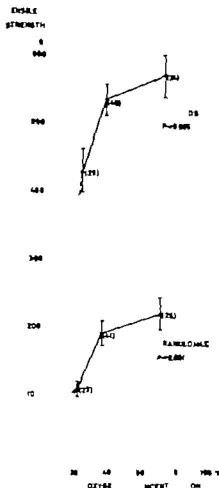


Fig 2 The average tensile strengths of the skin wounds and the granulomas (ten days after implantation) plotted against the actual oxygen concentrations in the breathing gas. The tensil strength of the skin wounds is given in g/cm of healing wound and of the granulomas i g/cm of the cross-sectional area. The number of experiments is indicated in the parentheses and the variation by the vertical bars which express the standard error of the mean. The statistical significance of th effect of the oxygen concentration is calculated by the analysis of the variances.

Chemical analyses on the granulomas have confirmed that the effect on the tensile strength depends on corresponding changes in the amounts of collagen hydroxy proline (unpublished work) The increased supply of oxygen may cause either an increased hydroxylation of proline, bound in collagen precursor or a less specific stimulation of the growth of the granuloma. Our data are not yet conclusive enough to decide between these two alternatives. A general effect of oxygen may be demonstrated, for example, in the higher content of nucleic acids in the granulomas from those rats which have been kept at th O_2-concentration of 35 or 70 %. How far the synthesis of collagen is regulated by the availability of oxygen for the hydroxylation of proline remains to be investigated

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Relationship Between Ventricular Pressures and Right and Left Myocardial Blood Flow

By

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Abstract

AUKLAND, K., F. KIIL and J. KJEKSHUS. *Relationship between ventricular pressures and right and left myocardial blood flow.* Acta physiol. scand. 1967. 70. 116—126.

The effects of constriction of the pulmonary artery and aorta on myocardial blood flow, measured as local hydrogen clearances, in the anterior parts of the right and the left myocardium have been studied in open-chest dogs. The increase in myocardial flow that by progressive constriction reached or exceeded 100 per cent of control values was in most experiments confined to the ventricle under increased pressure load. Plots of relative flows and pressures yielded regression lines of similar slopes for both ventricles without significant differences between systolic and mean ventricular pressures as indices of myocardial flow. Left myocardial flow remained unchanged when outflow resistance from the right ventricle increased until aortic pressure fell. The effect of increased aortic pressure on right myocardial flow was more variable. Increase of systolic left ventricular pressure to 250 mm Hg had slight or no effect on right myocardial flow. More severe aortic constriction, which doubled mean left ventricular pressure, increased right and left myocardial flow similarly. It is concluded that the relationship between relative flow and ventricular pressure variations previously demonstrated for the left ventricle also applies to the right ventricle and that myocardial flow is regulated independently for the two ventricles, except at extreme perfusion pressures.

It seems well established that the oxygen consumption of left ventricular contraction is mainly related to the development of myocardial tension (Sarnoff *et al.* 1958, Monroe 1964). Since the normally low oxygen tension in coronary sinus blood remains essentially unchanged when aortic resistance is increased, changes in left ventricular blood flow and oxygen consumption are closely correlated, suggesting that oxygen demand is the main regulator of myocardial flow (Braunwald *et al.* 1958). If these relations also applied to the right ventricle, myocardial flow would be distributed between the two ventricles according to their wall tension.

However, available information does not uniformly support this concept. Recent attempts to increase right ventricular pressures by constricting the pulmonary artery have either failed to increase right myocardial flow (Rosenblueth *et al.* 1959, 1961) or increased blood flow nearly as much in the left as in the right myocardium (Love and O'Meallie 1963). In other studies, increased right ventricular pressure in the face of a constant coronary perfusion pressure has been found to decrease right myocardial flow

suggesting that myocardial flow varies in proportion to the "coronary driving pressure" defined as the difference between aortic and ventricular mean pressures (Cross, Rieben and Salisbury 1961, Cross 1962). By perfusing both coronary arteries, Gregg *et al.* (1945) and Gregg and Shipley (1944) obtained a dual response to constriction of the pulmonary artery. In most of their studies inflow into both coronary arteries increased, but in some right coronary flow decreased, as if the increase in intra-myocardial pressure associated with an increase in right ventricular pressure opposed coronary flow.

The present study was undertaken in order to distinguish between these various possible determinants of myocardial flow. Myocardial blood flow was measured as hydrogen clearance (Aukland *et al.* 1967) from electrodes placed in the anterior parts of the right and left ventricles in open chest dogs. Ventricular pressures were varied over a wide range by constricting the pulmonary artery or aorta. By this technique it was also possible to examine whether increase in the outflow resistance of one ventricle influenced the myocardial blood flow of the other.

Methods

Mongrel dogs weighing 15—22 kg were anesthetized with sodium pentobarbital, 25 mg per kg i.v. with additional doses during the experiment which lasted 3—6 hrs. The trachea was intubated to allow the use of an AGA respirator and the chest opened by midsternal splitting. After excising the anterior part of the pericardium, silk sutures were placed along the incision line of the remaining pericardium and tied to the thorax wall in order to suspend the heart and prevent the lungs expanding over the anterior surface of the heart. Polyethylene catheters were inserted into the left and right ventricle and atrium through the auricular appendices, into the coronary sinus and into the ascending part of the aorta through the right carotid or femoral artery. Phasic and average pressures were recorded with Statham pressure transducers connected to Sanborn amplifiers and recorders. Graded occlusion of the aorta was produced either by inflating a balloon at the tip of a catheter introduced into the ascending limb of the aorta from a femoral artery or by means of a Blalock clamp placed on the ascending aorta. All occlusions of the pulmonary artery were performed with a Blalock clamp held in a fixed position throughout the experiment. Pulsus alternans was often seen when right or left ventricular pressure was suddenly increased. This alternans was usually transitory and could be prevented in subsequent experimental periods by increasing ventricular pressure slowly over several min. Transient pulsus alternans did not have any measureable influence on flows and pressures in the following control periods or on the response to constriction. L-shaped platinum electrodes for the recording of washout curves of hydrogen gas were placed in the myocardium of the left and right ventricle. Electrodes in the right ventricle were placed in the middle of the anterior surface and tentatively inserted into the middle layer of the myocardium. On the left ventricle the electrodes were placed at least one centimeter from the interventricular sulcus in the area between the anterior descending and the circumflex branches of the left coronary artery. The electrodes were inserted to various depths ranging from 1/4 to 3/4 of the myocardial cross-section. The observed flow did not vary consistently with the depth of impalement or with the localization on the anterior surface. No reference is therefore given to the exact position of the electrodes. The polarographic technique and the calculation of blood flow (ml/min/100 g) from the desaturation curves as well as the criteria for their acceptance has been described elsewhere (Aukland *et al.* 1967). Usually the recordings were started with [illegible] platinum electrodes in each ventricle. Rarely more than one of the inserted electrodes had to be excluded or reinserted on account of non-exponential desaturation curves or technical recording problems. When myocardial flow was recorded from two electrodes in one ventricle, the mean values are given. Following constriction of the aorta or the pulmonary artery at least [illegible] minutes elapsed before hydrogen gas desaturation curves were obtained. Periods of [illegible] were preceded and followed by flow measurements at control pressures. [illegible] experiments nearly [illegible] min elapsed between flow measurements in experimental and control periods. The exposed surface was prevented from drying out by [illegible] spray. At the end of the experiment the myocardium with the electrodes was excised and the position of the electrodes checked.

Cardiac output was measured in [illegible] experiments by the thermodilution technique using needle-shaped thermocouples inserted either in the pulmonary artery or in the femoral artery. Instantaneous

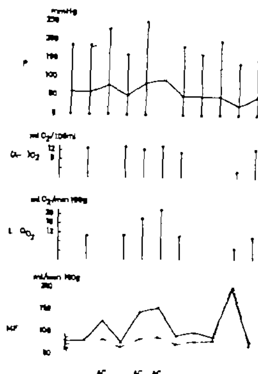


Fig 1 Effect of constriction of aorta (AC) and hypoxia (H) on left entricular pressure (LVP), myocardial oxygen extraction (A—V)O oxygen consumption (LV Q_{O_2}) and myocardial blood flow (MF) in left (L) nd right (R) entricle.

injections of 3—8 ml saline at room temperature were performed either in central vein or in the left ventricle.

For oxygen determinations arterial and coronary sinus blood, 4 ml was collected anaerobically in Luer glass syringes with the dead space filled with heparin solution Spectrophotometric determinations of hemoglobin concentration nd hemoglobin oxygen saturation ere performed as previously described (Aukland *et al* 1967)

Results

Constriction of the aorta

Left myocardial flow (LMF) The effects of aortic constriction on left, ventricular pressures (LVP) coronary arteriovenous oxygen extraction, m ocardial oxygen consumption and myocardial flow are shown in Fig 1 Aortic res stance was in this experiment increased in two periods, the first lasting 20 min and the second 60 min and three measurements of hydrogen clearance were obtained during increased aortic resistance. The increase in LMF was nearly proportional to the increase in LVP In sp te of an increase in systolic LVP to more than 250 mm Hg for an hour release of the aortic constriction resulted in return of myocardial flow to control values in less than 20 min. Arteriovenous oxygen differences remained constant, and the myocardial oxygen consumption increased therefore n direct proportion to myocardial flow The maximal increase in LMF was less than could be induced by arterial hypoxia (Fig 1 last experimental period)

The effect of increasing aortic resistance on LMF was similar in all experiments performed on 8 dogs. The relation between variations in LMF and mean LVP is

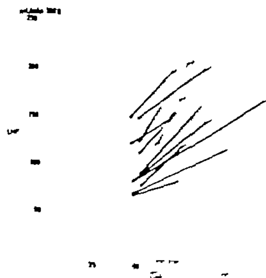


Fig. 2 Effect of constriction of aorta on relationship between left myocardial flow (LMF) and mean left entricular pressure (LVP)

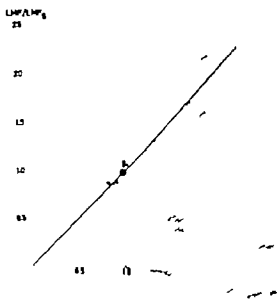


Fig 3. Relationship b tween relativ changes in left myocardial flow (LMF/LMF) nd relativ changes in mean left ventricular pressure (LVP/ LVP$_o$) U broken line indicates proportional changes, and large open circle (1 1) control values (LMF$_o$, LVP$_o$)

shown in Fig 2. Control mean LVP varied slightly wh...
varied widely between animals, with a range from 60 to ...
striction of the aorta mean LVP was increased by 35...
LMF w th pressure was almost as large at low as at hig... ...ac
of the wide scatter of flow values, the variations in LMF ... ...in
values (LMF and LVP were examined. Fig 3 shows ... ...ac
relati variations in LMF and mean LVP On accou... ...ed
true relationship between myocardial flow and pressu... ...ol

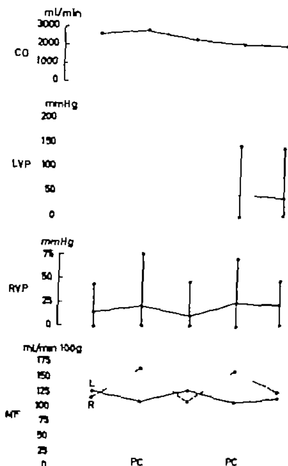


Fig. 4. Effect of constriction of the pulmonary artery (PC) on cardiac output (CO) left ventricular pressure (LVP) right ventricular pressure (RVP) and myocardial flow (MF) of the right (R) and left (L) ventricle.

LVP is greatly increased, but all data are included in the following calculations. The general equation is

$$y-1 = b(x-1) \tag{1}$$

where y = LMF/LMF and x = LVP/LVP and b is the slope of the line. With n sets of observations (x_i, y_i) the slope b and its standard error σ_b were estimated according to Hald (1952) as

$$b = \frac{\Sigma(y_i-1)(x_i-1)}{\Sigma(x_i-1)} \qquad \sigma_b = \left[\frac{1}{n-1}\left(\frac{\Sigma(y_i-1)}{\Sigma(x_i-1)} - b^2\right)\right]^{\frac{1}{2}}$$

Using data obtained during aortic constriction (Fig 3 filled circles) b = 0.833 ± 0.059. The relationship between the fractional changes of LVP and LMF when systemic pressure dropped during excessive constriction of the pulmonary artery is also shown in Fig 3 (open circles) When these data are included, b 0.817 ± 0.035. This is significantly less ($p < 0.05$) than unity (which indicates proportional changes in

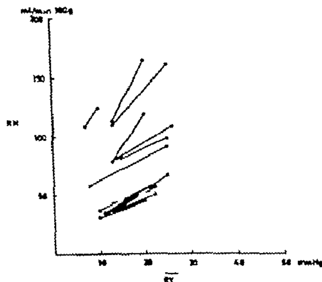


Fig. 5. Effect of constriction of the pulmonary artery on relationship between right myocardial flow (RMF) and mean right ventricular pressure (RVP) in compensated hearts.

myocardial flow and ventricular pressure) (Fig 3 unbroken line) The fractional resting myocardial flow' is estimated as the positive intercept at zero pressure, and was found to be 17 ± 7 per cent of control flow

Right myocardial flow (*RMF*) In the experiment shown in Fig 1 RMF remained nearly constant during increase of systolic LVP from 170 to 250 mm Hg indicating nearly complete autoregulation during increased perfusion pressure. The reliability of the measurements is demonstrated by the indentical increases in recorded RMF and LMF during hypoxia. Similar complete or nearly complete autoregulation was observed in 8 experimental periods on 3 dogs. By further elevation of mean LVP (up to 180 per cent of control values) RMF increased. The ratio RMF/LMF was reduced during constriction in 5 of 6 experiments on 3 dogs. Complete absence of autoregulation with increase in the ratio RMF LMF during constriction was observed in another dog when mean LVP was increased respectively to 200 and 248 per cent of control values. In the latter study cardiac output was reduced by more than 50 and RVP was slightly reduced In 3 animals RVP increased during aortic constriction presumably secondary to increase in left atrial pressure In these experiments the increase in RMF was not significantly larger than that obtained by increasing RVP to similar values by constricting the pulmonary artery

Constriction of the pulmonary artery

Right myocardial flow The effects of constriction of the pulmonary artery on cardiac output, ventricular pressures and myocardial flow in one experiment are shown in Fig 4 An increase in systolic RVP to 75 mm Hg had no influence on cardiac output LVP and LMF whereas RMF increased The results of all studies obtained in compensated hearts of 8 animals are shown in Fig 5 In spite of similar control

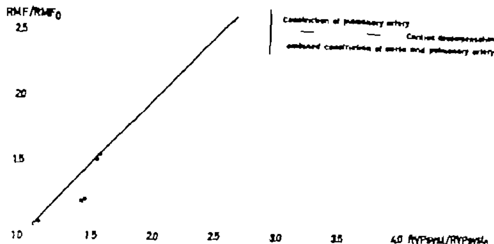


Fig. 6. Relationship between relative changes in right myocardial flow (RMF/RMF$_0$) and relative changes in systolic right ventricular pressure (RVP syst/RVP syst$_0$). Unbroken line indicates proportional changes, and the *point* (1 1) *control values.*

pressures, RMF varied widely between animals. An increase in mean RVP was associated in all studies with an increase in RMF

The relative variations in right ventricular flow and pressure are shown in Fig. 6 (filled circles) and were evaluated using equation 1. Right systolic pressure correlated ... better than mean pressure with flow. The slope of the regression line was ...±0.06. This is different from unity ($p<0.01$). The positive intercept at zero pressure was 21.5±7 per cent of control flows. Intercept and slope are not significantly different from those of the regression line for the left ventricle.

In four experiments additional constriction of the pulmonary artery led to large dilatation of the right ventricle with decreasing aortic pressure and reduction of RMF in spite of maintained or increasing RVP (Fig. 6, open circles). In one of these ... aortic and coronary pressures were increased above control values by constriction of the aorta. RMF increased, but right ventricular decompensation was not alleviated.

Left myocardial flow. Constriction of the pulmonary artery had no effect on LMF as long as systemic pressure was maintained. LMF did not increase significantly in any experiment, but was reduced when, as a result of severe constriction, cardiac output and systemic pressure were reduced. The relationship between the relative changes ... and LVP (Fig. 3, open circles) is expressed by a regression line with slope ... that is not significantly different from the slope of the regression line ... when LVP was varied by increasing aortic resistance.

Discussion

... measurements of myocardial flow by means of the hydrogen clearance technique show ... myocardial flow increases with ventricular pressures and that the increase in ... flow is largely confined to the ventricle under increased pressure load. ... flow ... increased pressure varied considerably

between animals, probably as a consequence of variations in hemoglobin concentration. This source of variation was eliminated by using relative rather than absolute values of myocardial flow.

For the left ventricle a linear relationship between myocardial flow and ventricular pressure has previously been established both by measuring flow into the coronary arteries (Gregg and Shipley 1944) as well as by drainage of blood from the coronary sinus (Braunwald et al. 1958). The oxygen cost as well as the coronary flow is probably mainly determined either by the maximal myocardial tension or the rate of development of tension, since the oxygen cost of the ejection period and the subsequent period of myocardial relaxation seems to be low (Monroe 1964). As myocardial tension is the product of geometric and pressure factors, a close relationship between ventricular pressures and myocardial flow would however only be expected if ventricular dimensions remained essentially unchanged at variations of outflow resistance. Several investigators, from Anrep (1912) to Taylor, Cingolani and McDonald (1966) have shown that increased aortic resistance has little or no influence on left ventricular dimensions as long as heart rate and stroke volume remain constant. Similar data for the right ventricle during obstruction of the pulmonary artery are not available. However, the observation that plots of relative values of ventricular pressures yielded regression lines of similar slope for both ventricles, would suggest that geometric factors, which may have different absolute values for the two ventricles, remained essentially constant during each experiment.

Mean and systolic ventricular pressure correlated equally well to myocardial flow. This finding is, for the left ventricle, in good accordance with previous studies. As long as diastolic pressures are negligible, mean ventricular pressure is proportional to the time tension index defined as the area under the systolic portion of the aortic curve (Sarnoff *et al.* 1958). Systolic ventricular pressure is closely correlated to the peak developed tension index (Monroe and French 1961). Both these indices would, under the present experimental conditions, correlate nearly equally well with oxygen consumption of the left ventricle, according to the data of McDonald, Taylor and Cingolani (1966). The present studies would therefore suggest that the concept of myocardial tension as a major determinant of myocardial flow is valid both for the left and the right ventricle. However, it has recently been demonstrated that the rate of development of tension as judged from the rate of development of ventricular pressure (maximal dp/dt) is a more important determinant of myocardial flow and oxygen consumption than the average or maximal tension (Sonnenblick *et al.* 1965; Kjekshus and Bugge-Asperheim 1966). In our studies, dp/dt was not measured, but it has previously been shown that systolic ventricular pressure and maximum dp/dt are highly correlated during variations in outflow resistance (Gleason and Braunwald 1962). Under the present experimental conditions it is therefore reasonable to assume that ventricular pressures and maximum dp/dt would correlate equally well to myocardial flow.

It was not possible to increase myocardial flow so much by increasing the outflow resistance as by arterial hypoxia. By marked constriction of the pulmonary artery

leading to large reductions in cardiac output and a fall in aortic pressure RMF might be reduced in spite of maintained RVP However in these experiments ventricular decompensation developed with large increase in right ventricular volume and increasing right atrial pressure. Vascular resistance was evidently not further reduced during decompensation since in 3 of 4 experiments the distribution of myocardial flow between the ventricles was not more in favour of the right ventricle than before decompensation developed. So far our results do not contradict the hypothesis advanced by Fineberg and Wiggers (1936) that right ventricular decompensation produced by constriction of the pulmonary artery is due to myocardial hypoxia, but an increase in aortic pressure that increased RMF considerably did not improve right ventricular function.

The finding of a correlation between RVP and RMF in the compensated heart is at variance with several previous studies. In studies on the isolated heart, Rosenblueth *et al.* (1959 1961) found that RMF remained unchanged when RVP increased On the basis of these and their own experiments, Sarnoff and Mitchell (1963) concluded that an increase in coronary perfusion is not a necessary concomitant of the increase in myocardial contractility at increased resistance to ventricular ejection However since a low coronary oxygen extraction is regularly observed in isolated preparations, increased oxygen demand might be met by increasing oxygen extraction rather than by increasing myocardial flow In studies with a cannula tied into the right coronary artery Gregg *et al* (1943) observed in some experiments a decrease in coronary flow during constriction of the pulmonary artery and suggested that the increased myocardial pressure opposed coronary flow Their and our studies are not directly comparable, since their observation periods lasted only a few min. With our technique transient changes in flow until a new steady state is reached would not be discovered. Similarly the finding of a high correlation between myocardial flow and coronary driving pressure (Cross *et al* 1961) might reflect non-steady state conditions. In most of our studies mean coronary driving pressure" calculated as the difference between integrated aortic and ventricular pressures did not even show the same directional variations with myocardial flow for both ventricles.

Under extreme aortic constriction, which more than doubled mean LVP RMF sometimes increased more than LMF as would be expected if coronary blood flow was controlled by the coronary driving pressures rather than by the ventricular pressures. Under more physiological conditions, RMF did not increase with aortic pressures. It is not possible from the present experiments to define the range of right myocardial autoregulation, since aortic obstruction mainly increased systolic and mean aortic pressures but had only a slight effect on diastolic aortic pressure. It is clear however that RMF *was not measurably influenced* by moderate aortic obstruction, either directly as a consequence of more forceful left ventricular contractions, or indirectly through changes in chronotropic and inotropic stimuli mediated through changed baroreceptor activity

Our studies clearly showed that an increase in RVP had no effect on LMF that was not related to variations in LVP This finding of a selective increase in RMF

during pulmonary constriction is at variance with previous studies. Gregg *et al.* (1943) observed a rise in left coronary inflow during pulmonary constriction. As suggested by these authors, this increase might not reflect increased LMF since both the circumflex and the descending branches of the left coronary artery perfuse parts of the right ventricle. More difficult to reconcile with the present findings are the observations of Love and O'Meallie (1963) who found in Rb[4] clearance studies that constriction of the pulmonary artery increased LMF. It has been claimed that stretching of the pulmonary artery may increase systemic resistance and therefore in the face of a maintained cardiac output increase aortic pressure (Lewin *et al.* 1961) and it has also been claimed that partial occlusion of the pulmonary artery increases cardiac output (Taquini and Aviado 1961). Neither cardiac output nor systemic resistance increased in our studies. Furthermore, a slight increase in LVP would not explain an increase of 50 per cent in LMF as was found in the rubidium clearance studies during pulmonary constriction. The considerable variation in control flow between dogs at similar ventricular pressure, however, makes the results obtained with the rubidium clearance technique difficult to interpret, since this technique does not permit of using the dogs as their own controls.

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Influence of K ions and Adrenaline on the Adenosine 3', 5'-monophosphate Content in Rat Diaphragm

By

Lennart Lundholm, Theodore Rall and Sandor Vamos

We have found that the phosphorylase activating effect of adrenaline and isoprenaline in isolated rat diaphragm was blocked when the muscle was suspended in a high-K solution (Lundholm, Mohme-Lundholm and Vamos 1967). The phosphorylase-activating effect of the catecholamines in skeletal muscle is associated with stimulation of the production of adenosine 3', 5'-monophosphate (cyclic AMP) (Sutherland and Rall 1960). The content of this nucleotide in skeletal muscle is also increased *in vivo* by adrenaline (Posner, Stern and Krebs 1962, 1965).

The causative factor in the blockade of the phosphorylase activating effect of adrenaline in high-K solution was assumed to be one or both of the following alternatives. *1* The stimulation by adrenaline of the cyclic AMP production was inhibited in high-K solution. *2* The effect of cyclic AMP on the phosphorylase activation was blocked in high-K solution. In the study described below the first alternative was investigated.

A rat diaphragm was divided into four parts. Two of these parts were suspended in normal Krebs bicarbonate buffer bubbled with 5 % CO_2 in O_2 at 37° C and the other two were suspended in a Krebs bicarbonate buffer in which all Na had been replaced by K, the K concentration being 143 meq/l. After a preincubation period of 30 min adrenaline in a concentration of 1 · 10⁻ g/ml was added to one of the specimens in the normal solution and one in the high-K solution. After a further 5 min all specimens were analysed for their content of cyclic AMP. This content was determined by Rall's modification (to be published) of the method of Butcher *et al.* 1965.

The results are given in Table I. In the control specimens the cyclic AMP content was on the average 0.21 nmoles/g wet weight and after the addition of adrenaline this content increased about 15-fold. In the specimen from the high-K solution the cyclic AMP content was about nine times greater than in that from the normal Krebs solution. Adrenaline also had a strong stimulatory effect on the cyclic AMP content of the specimen from the high-K solution and in 6 out of 8 expts this content exceeded the adrenaline effect in the normal solution. However, no potentiation of

TABLE I Influence of adrenalin (10^{-4} g/ml) and K (145 meq/l) on the content of cyclic AMP (nmoles/g wet weight) i isolated rat diaphragm. Mean ± S.E.M. P = probability that th effect was due to chance. = number of tests.

Control	K	Adrenaline	K + adrenaline
0.21 ± 0 05	1.9 ± 0.06	3 17 ± 0.89	6 17 ± 0.55
n = 9	= 9	n = 8	= 9

Difference between		
	K nd control	= 1 72 ± 0.29 P < 0.001
	Adrenaline and control	= 2.93 ± 0.88 P < 0.01
	(K + adrenaline) and K	= 4.05 ± 0.40 P < 0.001

the adrenaline effect in the high K solution could be statistically proved instead the K and the adrenaline seemed to produce a summated effect.

These results showed that the stimulation of the cyclic AMP production in skeletal muscle by adrenaline was not blocked in high K solution. This would suggest the *possibility that the stimulating effect of cyclic AMP on phosphorylase a activity* was somehow blocked in high-K solution This interpretation would also explain the low phosphorylase *a* content of diaphragms incubated in high K solutions alone (Lundholm, Mohme Lundholm and Vamos 1967) even though the cyclic AMP content was markedly elevated (Table I)

The increased cyclic AMP content observed in high K solutions cannot be explained readily Posner Stern and Krebs (1965) observed that tetanic stimulation of rat gastrocnemius muscle *in situ* did not result in elevated cyclic AMP content, even though phosphorylase *a* content and phosphorylase *b* kinase activity was increased. Thus t does not seem possible to ascribe the changes in cyclic AMP content in high-K solutions simply to depolarization of the membrane.

The additative effect of adrenaline and K on the cyclic AMP content may indicate that different receptors of adenylcyclase or different kinds of this enzyme was stimulated. The mechanism underlying the increased cyclic AMP accumulation in muscle exposed to high K solutions will be the subject of further studies.

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Muscle Glycogen and Muscle Electrolytes during Prolonged Physical Exercise[1]

By

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Abstract

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Nine volunteers have been examined during prolonged physical exercise to exhaustion at a load of about 60 per cent of $\dot{V}_{O_2\,max}$. Ordinary circulatory parameters were measured as well as the quantities of glycogen, water and electrolytes in muscle tissue obtained by needle biopsy. In a separate study 6 subjects were examined for respiratory quotient under similar exercising conditions. The muscle glycogen fell considerably from a mean of 6.9 per 100 g glycogen and fat-free solids to a mean of 1.7 g at the end of exercise. The quantity of muscle glycogen used was correlated both to total energy developed during exercise and also to duration of exercise.

The electrolyte and water content in muscle tissue showed only small changes. Some increase was found in muscle sodium and chloride and also in the chloride space. The potassium content fell significantly by about 4 per cent of the basal value. None of the circulatory parameters measured showed changes of such magnitude as to have a limiting effect on performance. Two subjects were examined also with glucose infusion during the exercise. The reduction of glycogen, as also the performance of these two subjects, was of the same order of magnitude with and without infusion of glucose. The results suggest that the capacity for prolonged work is directly correlated to the glycogen store in the working muscles.

The utilisation of energy during physical exercise has been studied for many years. The energy needed for muscular work is taken chiefly from fat and carbohydrate depots. The role of fat metabolism in energy production has been reported by several authors (Krogh and Lindhard 1920, Christensen and Hansen 1939). In recent years the isotope labelling technique has permitted direct studies of the metabolism of fatty acids (Havel, Naimark and Borchgrevink 1963, Havel *et al.* 1964). Determination of the RQ has revealed that the part of the energy requirement that is taken from the carbohydrate depots increases with rising intensity of work (Christensen and Hansen 1939).

[1] A preliminary report on muscle glycogen during prolonged exercise was delivered at the Annual Meeting of the Swedish Medical Society, Clinical Physiological Section, in November 1964.

However the methods used in all these studies of the part played by carbohydrate metabolism in energy production are indirect the conclusions having been drawn after calculation of the RQ. No direct measurements of the carbohydrate depots and their relation to physical exercise have been made on man. In recent years, however the needle biopsy method has provided a means of obtaining muscle tissue which can be analysed for its content of electrolytes and water (Bergström 1962) and glycogen (Bergström and Hultman 1966 a, b Hultman 1967 a b). This method was used in the present investigation to study the influence of heavy prolonged physical exercise on the content of glycogen, water and electrolytes in working muscles.

The subjects were subjected to continuous exercise on a bicycle ergometer until they had to give up on account of exhaustion

Methods

Material

The subjects were 15 healthy young volunteers. Nine took part in the biopsy experiments, the other six were examined for RQ. They varied degree of physical training the majority being normal by Swedish standards. Anthropometrical data and certain data pertaining to the circulatory system re given in T ble I Prior t the experiments they were subjected to physical examination nd to exercise tests with electrocardiographic recording t rest and during exercise. No subject showed any sign of disease nor any circulatory bnormality Nor did any subject exhibit any pronounced orthostatic reaction. The relations between physical working capacity heart olume and total haemoglobin were normal according t data given by Holmgren, Jonsson and Sjostrand 1960.

Methods of determination

Th physical *working capacity* was expressed as the rate of work (kpm/min) the subject could perform on bicycle ergometer t a heart rat of 170 beats per min (W_{170})

The electrocardiogram, total mount of haemoglobin, blood olume heart volume blood pressure nd the respiratory quotient were determined according to the methods employed by Ekelund and Holmgren (1964)

Lactic acid concentration was determined according to Barker and S mmerson as modified by Ström (1949)

Potassium and sodium in blood nd urine were measured with Eppendorff's flame photometer

Chloride in blood and urine was determined by Shales and Shales t tration method as modified by Brun (1949) *phosphorus* by the method of Gomori (1941)

Glucose in blood was nalysed by the orthotoluidine method (Hultman 1959

Protein in blood was nalysed by the Biuret method.

Muscle biopsies from *m quadriceps f moris* of the righ and left legs l eratel were performed with biopsy needle The biopsy technique nd the weighing of the pieces of muscle has been earlier described detail (Bergström 1962) At every biopsy tw samples were aken for glycogen nalysis and one or two for determination of water nd electrolyt conten

For *glycogen* analysis weighed muscle tissue (5—10 mg) as homogenized cold er l Potter El hjem glass-homogenizer The protein in the homogenate was prec pitated with trichloroacetic acid. The time delay between the biopsy nd the protein precipitation as less than 10 min. After centrifugation the glycogen n the protein-free supernatant was prec pitated h ethyl alcohol (10 vol of alcohol t l vol of supernatan) After 12 h ice bath he gl ogen precipitate was centrifuged, and the superna ant removed. The precipitat was h drol zed th $1 N$ sulfuric acid i an oven t 120 C for 3 h. The glucose formed was nalysed by he o toluidine method. A glycogen preparation was tre ed in he same w y nd used as standard The mean alue of the glycogen content in *m quadriceps femoris* in 228 normal subjects as 1 39 g per 100 g et muscle (96 per cen of the alues were bet een 0 93 nd 2 0 g per 100 g Standard error of the method calculated on duplicate biopsies (S D.= $\sqrt{\Sigma d^2/2n}$) was 0 070 g per 100 g tissue I his series the mean alue from duplicat biopsies was used, and the standard error was reduced to 0 030 g per 100 g tissue A detailed description of his method is in press (Hultma 1967 b No significant difference was found between the proximal and the distal part of the *quadriceps femoris* muscle or between left and right leg muscle i the same subject

TABLE I Some anthropometric data in 9 subjects examined as t muscle glycogen and in 6 subj ct as to RQ t long-term exercise

Subject	Age (yrs)	Height (cm)	Weight (kg)	W_{max} (kpm/min)	Heart volume (ml)	Total haemoglobin (g)	Blood volume (l)
1	23	181	87	1 700	1 180	810	5 7
2	23	1 4.5	70.5	1,600	1,010	775	5.9
3	1	176.5	64 5	1,250	730	700	5 4
4	21	175	62.7	1,200	700	675	5.6
5	22	171	67 1	750		65	5.1
6	22	168	56	900			
7	21	180	73.2	1,000	685	763	4 7
9	22	187	71.0	1,050	810	775	5 6
10	24	177.5	69.2	950	745	645	4 8
(RQ 1)	(22)	(172.0)	(61.6)	(1,050)	(730)		
(RQ 2)	(22	(183 0)	(80 9)	(1,350)	(870)		
(RQ 3)	(21)	(190.0)	(83 3)	(900)	(780)		
(RQ 4)	(21)	(170.5)	(71.3)	(1,250)	(920)		
(RQ 5)	(20)	(186.0)	(70 6)	(1,200)	(880)		
(RQ 6)	(20)	(179.0)	(72.0)	(1,300)	(860)		
–	22.1	176 7	69 0	1 156	840	739	5 4
	(22 0)	(180)	(73 3)	(1 175)	(840)		
Range	21—24	168—187	56 0—87.0	750—1 700	685—1 180	645—810	4.7—5.9
	(20—22)	(172—190)	(61 6—83 3)	(900—1,350)	(730— 920)		

The biopsy specimens for ater and electrolyte determination (5—1 mg) ere nalysed by previously described methods, neutron activation analysis being used for determination of sodium, potassium, chloride and phosphorus (Bergstrom 1962).

The chloride space hich is considered to be comparati ely good measure of the content of extracellular fluid in the muscle tissue as calculated (Manery 1954 Bergstrom 1962 The quantity of sodium not accounted for in this space, he excess sodium total sodium in muscle minus sodium in the hloride space was used as measure of the intracellular sodium content.

W ter and electrolytes in muscle ere related to glycogen- nd fa -free solids (GFFS) i.e. the part of he dry eigh hich consisted of gly ogen as subtracted from the fat-free dry eight. The reason for this as tha the reference basis used should not hange in the course of the experiment GFFS consists partly of the dry substance of the muscle cells, chiefl cellular protein, and partly of the extracellular connecti tissue Earlier in estigations ha show that the protein catabolism during muscula ork is mall (Christensen and Hansen 1939 and it is also improbable that the connecti issue content hanges during this type of experiment.

Experimental routine

By means of prior model experiments the subjects ere made familiar h the form of exercise and the load hich as performed The intention as tha the exercise should ontinue until the subjects had to gi up on account of exhaustion I the biopsy experiments the load hich the exer ise as performed as on an average 62 per ent of W T of the subjects repeated the same test some 6 months later under ontinuous infusion of 20 glucose solution Prior to the tests he subject had fasted for hour 1 h After percutaneous insertion of theters into brachial artery basal blood alues ere obtained Biopsy as performed in m quadriceps femoris and there after the bic cle ergometer exercise tarted At the end of the exercise and one hour later new muscle biopsies ere performed

Urine as ollected for he determination of electrolytes in 8 of the biopsy experiments ithout glucose infusion, both during period 1 – h before the exercise and again after he conclusion of he exercise The ollection times ere noted and the olume of urine measured

During the exercise the subjects ere allowed to drink ter as desired

TABLE II Load, duration, and muscle glycogen at long term exercise

Subject	Work load kpm/min W_{max}	absolute	% of W_{max}	Duration min	Developed energy kpm × 10^4	A g glycogen per 100 g fat free and glycogen free solids: At rest	3' after the end of exercise	decrease from the resting value
1	1 700	1 000	59	127	12.7	6.7	2 0	4 7
2	1,600	900	56	190	17 1	11 1	3 6	7.5
3	1,250	790	63	135	10 7	8.2	2 0	6.2
(3G)	(1,250)	(700)	(56)	(165)	(11.6)	(5 4)	(1 6)	(3 8)
4	1,200	800	67	112	9 0	6.5	1 3	5 2
(4G)	(1,200)	(900)	(75)	(90)	(8.1)	(6.4)	(1 0)	(5.4)
5	750	450	60	70	3.2	6.4	2 4	4 0
6	900	550	61	92	5 1	4 7	1 0	3.7
7	1 000	650	65	116	7.5	6.9	0.7	6 2
9	1 050	650	62	120	7.8	5 3	0 9	4 4
10	950	600	63	180	10.8	6.9	1 6	5.3
n	9	9	9	9	9	9	9	9
$\bar{x}$	1 156	710	61.8	126.9	9.3	7 0	1 7	5 24
S D	319	176	3.3	38.3	4 1	1 8	0 9	1.22
S.E. of the mean						0.6	0 3	0.41

Subjects 3G and 4G not included in the statistics

Results

A. Without glucose administration (9 subjects)

The 9 subjects who took part in the biopsy experiments exercised at an average load of 62 per cent (range 56—67) of W_{max} corresponding to 710 kpm/min (range 450—1 000) — see Table II. The average exercising time was 127 min (range 70—190). To obtain a measure of the total energy developed during the exercise the load was multiplied by the worktime. The average quantity of energy developed was 9.3 × 10^4 kpm (range 3.2 × 10^4 — 17.1 × 10^4).

The corresponding figures for the 6 subjects in which RQ determinations were made were load 71 per cent of W_{max} (range 62—78) corresponding to 830 kpm/min (range 650—950), exercising time 121 min (range 90—135) and quantity of energy developed 10.1 × 10^4 kpm (range 7.7 × 10^4 — 12.4 × 10^4). The figures reported in the following, except for RQ, refer to the 9 subjects in the biopsy experiments.

		B. g glycogen per 100 g wet muscle				
60' after the end of exercise		At rest	5' after the end of exercise		60' after the end of exercise	
	increase from 5' after the end of exercise			decrease from the resting value		increase from 5' after the end of exercise
1 7	−0.5	1.30	0.45	1 05	0.39	−0 06
4 4	0 8	2.43	0.80	1.63	0.98	+0.18
1.8	−0.2	1 80	0.45	1.35	0.41	−0.04
(3.4)	(1.8)	(1.22)	(0.33)	(0.89)	(0.74)	(0.41)
1 4	0.1	1 48	0.30	1 18	0.30	0 00
(2.3)	(1.5)	(1 37)	(0.30)	(1.07)	(0.50)	(0.20)
2.6	0.2	1.27	0.49	0 78	0 61	0 12
0 8	−0.2	1.01	0.23	0 78	0.19	−0.04
1 4	0.7	1.55	0.14	1 41	0.28	0.14
0 4	0.5	1 19	0 19	1 00	0 09	−0.10
1 8	0.2	1.55	0 37	1 18	0.41	0.04
9	9	9	9	9	9	9
1.8	0 09	1.35	0.38	1 15	0.41	0.03
1 2	0 44	0.41	0.20	0.28	0.26	0.10
0 4	0.15	0.14	0.07	0 095	0 09	0 03

The *heart rate* rose successively during the continuous exercise in all the subjects Fig 1)

The respiration also increased gradually during the exercise period (Fig 1)

Blood pressure (in 6 subjects) was measured in a brachial artery (Fig 1) The *mean blood pressure* increased during the first 10 min of exercise and returned thereafter successively towards the basal value (Fig 1)

The *blood volume* measured with I[131] averaged at rest 5 6 1 (range 4 6—6.9 n = 5) and after 10 min of exercise 5.3 l range 4 4—6 0 n = 5) Blood volume at rest, obtained with I[131] agreed closely with those obtained by the COHb method (5 6 and 5 3 l respectively)

The *lactic acid* concentration at rest averaged 0 64 meq/l. After 15 min of exercise it had risen to 2.18 meq l It then decreased and remained lower during the rest of the work period Fig 2) The 15 and 30 min values both differed significantly from

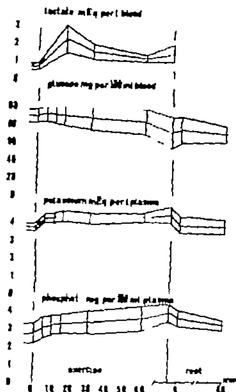


Fig. 1

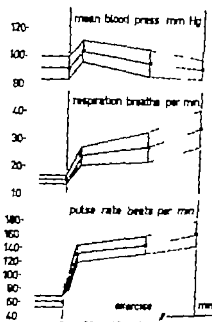


Fig. 2

Fig. 1 Some blood constituents before, during, and after exercise. Mean value and standard deviation in 9 subjects.

Fig. 2 Some physiological parameters before during, and the end of exercise

the value at rest ($p < 0.001$) Between the 30 and 60 min values there was an almost significant difference ($p < 0.05$)

Plasma protein averaged at rest 7.0 g per cent (range 6.6—7.5). Five min after the end of exercise, the protein concentration averaged 7.4 g per cent (range 6.9—8.1). Sixty min after the end of exercise the average was 7.1 g per cent (range 6.7—7.6). There was a significant difference between the initial value and the values at both 5 and 60 min after exercise ($p < 0.01$)

The *sodium* concentration in plasma averaged at rest 139 meq/l (range 132—145) 5 min after the end of exercise 143 meq/l (range 134—148) and 30 min after exercise 143 meq/l (range 138—145). There was a significant difference between the values at rest and 5 min after the end of exercise ($p < 0.01$)

The *chloride* concentration in plasma averaged at rest 107 meq/l (range 104—110) 5 min after the end of exercise 107 meq/l (range 102—111) and 60 min after exercise 106 meq/l (range 103—109)

The *plasma potassium* concentration increased during exercise (Fig. 2). The main rise occurred within the first 5 min. Significant differences ($p < 0.001$) were found

TABLE III Water and electrolytes in muscle tissue before and after exercise
Muscle constituents per 100 g glycogen-free fat-free solids

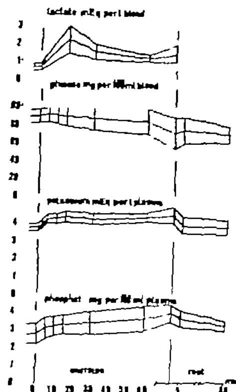
Subject		H_2O g	H_2O_e g	H_2O_i g	Cl mEq	Na mEq	Excess Na mEq	K mEq	P mM
1	Before	342	54	288	6 4	9.2	1.8	47.3	32.8
	After	335	54	281	6.4	9.0	1.2	43 6	30.0
2	Before	344	49	295	5.9	7.6	0.9	47.8	32.4
	After	341	52	289	6.3	8.9	1.5	46.2	32.0
3	Befor	342	53	289	6.3	9 1	1 7	46.4	32.0
	After	352	85	264	10 4	11.6	0	46.4	32.4
(3G)	Before	(333)	(51)	(282)	(6.0)	(9 1)	(2.1)	(44 4)	(28 1)
	After	(372)	(75)	(297)	(9.0)	(12.6)	(2.2)	(41 6)	(27 4)
4	Before	348	50	298	5 9	8.1	1.2	45.5	—
	After	372	64	308	7.6	9 9	0 7	44 8	—
(4G)	Before	(352)	(49)	(303)	(5 7)	(8.8)	(2.2)	—	—
	After	(345)	(52)	(243)	(5.9)	(9.6)	(2.9)	—	—
5	Before	387	—	—	—	—	—	—	—
	After	363	—	—	—	—	—	—	—
6	Before	352	48	304	5.9	10 2	3.6	44 6	30.0
	After	351	57	294	7 1	10.0	1.9	44 1	30 4
7	Before	338	32	306	6 1	9 8	4	48 6	31.2
	After	369	70	299	8 4	12.6	2.2	45 8	28.8
9	Befor	354	51	303	6 2	8 8	1 4	50 1	31 1
	After	363	63	300	7 1	9 2	0	46.3	30.4
10	Before	359	48	311	5 7	9 1	2 1	51 1	33.3
	After	363	51	312	5.9	10 1	2 7	47 1	31 1
	Befor	334.0	30 6	299 3	6.03	8.99	1.89	47.65	31 8
$\bar{x}$	After	356.8	61 8	293 4	7 40	10 16	1 28	45 54	30 7
	Difference	2.8	11 2	5.9	1.35	1 17	—0 61	— 2 11	1 1
	p	0 05	0 01— 0 05	0 05	0 01 0 05	0 01— 0.05	0 05	0 001— 0.01	> 0 05

(Difference After Befor)
Subjects 3G and 4G not included in the statistics

between the initial value and all other values up to 60 min of exercise. Five min after the end of exercise the potassium concentration did not differ significantly from the initial value

The *phosphorus* concentration in plasma also increased during exercise The main rise came during the first 60 min (Fig 2) Significant differences $p < 0.01$) were found, between the resting values and the values after 15 30 and 60 min of exercise at the end of exercise and 5 min after the end of exercise

Glucose As appears from Fig 1 the blood sugar was lowered at the end of exercise and in the period after exercise It was a significant difference between the blood glucose concentration before exercise and at the end of exercise $p < 0.001$ and 10 min and 60 min after exercise $p < 0.01$) respectively The lowest blood sugar



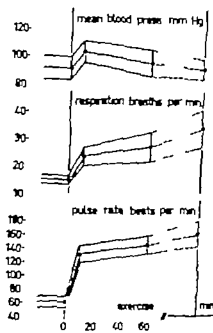


Fig 1 Some blood constituents before during, and after exercise. Mean value and standard deviation in 9 subjects.

Fig 2. Some physiological parameters before, during, and at the end of exercise.

the value at rest ($p < 0.001$) Between the 30 and 60 min values there was an almost significant difference ($p < 0.05$)

Plasma protein averaged at rest 7.0 g per cent (range 6.6—7.5) Five min after the end of exercise, the protein concentration averaged 7.4 g per cent (range 6.9—8.1) Sixty min after the end of exercise the average was 7.1 g per cent (range 6.7—7.6) There was a significant difference between the initial value and the values at both 5 and 60 min after exercise ($p < 0.01$)

The *sodium* concentration in plasma averaged at rest 139 meq/l (range 132—145) 5 min after the end of exercise 143 meq/l (range 134—148) and 30 min after exercise 143 meq/l (range 138—145) There was a significant difference between the values at rest and 5 min after the end of exercise ($p < 0.01$)

The *chloride* concentration in plasma averaged at rest 107 meq/l (range 101—110) 5 min after the end of exercise 107 meq/l (range 102—111) and 60 min after exercise 106 meq/l (range 103—109)

The plasma *potassium* concentration increased during exercise (Fig 2) The main rise occurred within the first 5 min. Significant difference ($p < 0.001$) were found

0.01). The increase of the chloride space and the increase of the chloride and sodium content were almost significant ($p < 0.05$).

Urinary water and electrolyte excretion. Compared with the control period, there was a significant fall-off in excretion per min of water, sodium, potassium and chloride during the exercising period. The phosphate excretion was not significantly changed (Table IV).

Water intake. During exercise the subjects were given an average of 1 l of water. The absolute loss of weight was between 1 and 2 kg.

B. *With glucose administration (2 subjects)*

In the glucose infusion experiments 8—10 ml of 20 % glucose solution was administered per min. The two subjects received 330 and 180 g of glucose, respectively. As regards heart rate, breathing rate and blood pressure, these experiments show no tendency to differ from the tests without glucose infusion. The blood glucose levels in the infusion experiments were 310 and 254 mg/100 ml after 60 min of exercise and 214 and 238 mg/100 ml, respectively, at the end of exercise.

Muscle glycogen showed a marked decline during exercise with glucose administration. The glycogen decrease was of the same order of magnitude as during work without glucose infusion. In the experiments with glucose infusion a clear tendency to restitution of muscle glycogen was observable in the first h after the end of exercise. See Table II.

C. *Studies of respiration quotient (RQ.)*

As stated, the RQ was not determined in conjunction with the biopsy experiments. The oxygen uptake ($n = 5$) was at rest 296.2 ml (range 254—367), after 15 min of exercise 2018 ml (range 1545—2250). After 60 and 120 min of exercise and at the end of exercise the values were, respectively, 2155 ml (range 1695—2405), 2315 ml (range 1900—2570) and 2349 ml (range 1880—2700). The mechanical efficiency after 15, 60, 120 min and at the end of exercise was, respectively, 23.1 per cent (range 21.0—24.1), 21.3 per cent (range 19.5—22.4), 19.6 per cent (range 17.7—21.2) and 19.3 per cent (range 17.7—21.0). The RQ at rest, after 15, 60 and 120 min of exercise and at the end of exercise was, respectively, 0.84 (range 0.80—0.89), 0.88 (range 0.86—0.91), 0.86 (range 0.85—0.87), 0.85 (range 0.80—0.86) and 0.82 (range 0.79—0.86). These figures agree with earlier reports (Ekelund and Holmgren 1964). The 15 min value is significantly higher than the resting value ($p < 0.05$) and also than the value at the end of exercise ($p < 0.01$).

Discussion

In animal studies it has been demonstrated that muscle glycogen is used during exercise, but no previous investigators have reported direct studies on man. We have earlier studied the effect of exercise on the muscle glycogen in man (Bergström and Hultman, 1966 a) by the same method of analysis but under partially different

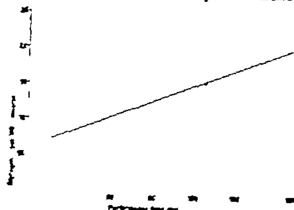


Fig. 3. The relationship between muscle glycogen concentration (g per 100 g wet muscle tissue) and work performance time in 9 subjects. Equation of regression line $y = 0.00732x - 0.[illegible]61$ $r = 0.68$ $p < 0.05$.

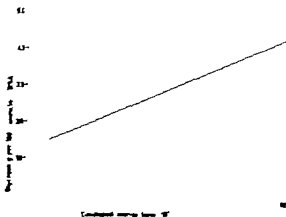


Fig. 4. The relationship between muscle glycogen concentration (g per 100 g wet muscle tissue)/body surface area (m[illegible]) and developed energy (kpm $\times 10^4$) in 9 subjects. Equation of regression line $y = 0.1[illegible]9x - 0.123$ $r = 0.87$ p [illegible] 0.01

experimental conditions. We found that even relatively light exercise caused a lowering of muscle glycogen, although not of the same order as in the present experiments. The effect of heavy exercise has also been studied in some cases, and reductions of glycogen to unmeasurable concentrations were observed (Bergström and Hultman 1966 b).

As regards circulatory factors during exercise the subjects showed a successive rise in pulse rate, continuous fall of mean arterial pressure and an increase in respiratory rate. There was also an initial reduction of the plasma and blood volume which thereafter remained unchanged during the exercise. Ekelund and Holmgren (1964) found similar circulatory changes after one h of cycling; the cardiac output was measured and found to be unchanged. They recorded an initial increase of the respiratory quotient at the start of exercise. As observed earlier (Christensen and Hansen 1939) the RQ fell at the end of the exercising period, indicating a reduction of the carbohydrate part of the metabolism. Under the present circulatory conditions the duration of exercise does not appear to be limited by circulatory or respiratory factors.

A close correlation was found between performance time and initial muscle glycogen content (Fig. 3). There was also a fair correlation between muscle glycogen

decrease and performance time ($y = 0.0047x + 0.53$ r 0 62 $p < 0.05$) The total amount of glycogen in the working muscles could not be calculated as the size of this muscle mass was not known. Still it should amount to the same proportion of the total muscle mass in all the subjects as they were doing the same type of exercise. It is known that there is a fair correlation between muscle mass and body surface area (BSA) (Talso *et al.* 1960) For these reasons we multiplied the glycogen values by BSA and correlated this product to the developed energy Close correlations were found both when initial glycogen concentration (Fig 4) and glycogen decrease were used ($y = 0.106x + 0.113$ r 0 76 $p < 0.02$)

All these correlations indicate that the local glycogen store in the working muscles is a determining factor for the ability to perform long-term exercise, i.e., the higher the muscle glycogen content, the longer the performance time. The reason why the performance diminishes when the glycogen concentration is low is not evident from these experiments. However it has recently been shown that the glycogen concentration is critical for the resynthesis of phosphorylcreatine and ATP in the working muscles in man (Hultman and Bergström 1967)

In Mc Ardle's disease phosphorylase is lacking in the muscle. This results in inability to utilize glycogen via phosphorylation, as reflected, *inter alia*, in the absence of lactic acid formation during exercise (Porte *et al.* 1966) These patients have a greatly reduced physical working capacity which furthermore reveals the important role of glycogen utilization in the development of muscular energy during exercise.

An attempt was also made to calculate the working muscle mass from the decrease in muscle glycogen, assuming that the utilized carbohydrates comprise only muscle glycogen. In the calculation a mean RQ of 0 84 and a mechanical efficiency of 21 per cent was applied. Using these figures, the average work performed should give the found mean decrease in muscle glycogen concentration in a muscle mass of 9 7 kg provided that the glycogen decrease was uniform in the working muscles. The corresponding muscle mass for an RQ of 0.81 should be 7.5 kg Both these figures seem to be low for bicycle work Still it must be remembered that this calculation rests on the assumption that the average decrease of glycogen in *m. quadriceps femoris* also takes place in the other working muscles of the leg P obably most of the other muscles worked with lower intensity and, consequently the glycogen decreases were smaller

Of the other physiological parameters measured during prolonged exercise none has shown changes of such magnitude as to have a limiting effect on performance A possible exception is the blood sugar which in two cases was considerably lowered at the end of exercise

A point of special interest in the two studies of exercise during glucose infusion was that the lowering of glycogen was of the same order as when the subjects exercised without administration of glucose despite the fact that the glucose content in the arterial blood in the former experiment was greatly elevated This suggests that the glycogen catabolism during exercise takes place at almost the same rate irrespective to the quantity of glucose delivered to the muscles i e there appears

to be an obligatory breakdown of muscle glycogen. It also suggests that the muscle glycogen content is an important limiting factor in the ability to perform exercise also when the blood glucose concentration is high. In studies of Havel *et al.* by means of C¹⁴-labelled fatty acid it was shown that the share of the free fatty acids in the energy metabolism during exercise was greatly reduced by carbohydrate administration (Havel *et al.* 1964). It thus appears as though the metabolism of added glucose replaces, to a great extent, the fat combustion during work, but cannot replace the energy derived from muscle glycogen.

During exercise water, sodium, potassium and chloride were lost both with the urine and by perspiration. The urine losses per minute, however, were less during than before the exercise. The increase of the sodium concentration in plasma despite unchanged chloride concentration might be due either to an egress of sodium from the cells or to a proportionally lower excretion of sodium than of chloride in perspiration and urine. It is known that the sodium chloride ratio in sweat is lower than in the extracellular fluid (Robinson and Robinson 1954). The same applied to the urine collected after exercise in our experiments. We did not find a definite loss of sodium from the muscle cells (reduction of excess sodium) during exercise. A rising concentration of sodium, potassium and phosphorus in plasma during exercise has been earlier reported by, among others, Ewig and Wiener (1928).

In the muscle tissue the chloride and sodium content and also the chloride space increased significantly ($p < 0.05$). This indicates an increased quantity of extracellular fluid in the working muscle provided that no chloride shift takes place across the cellular membranes. An increase of water locally in isolated muscle has been reported in conjunction with muscular activity (Fenn 1936, Sréter 1963). The increase consisted of accumulated extracellular fluid (measured on the basis of inulin space), whereas the intracellular fluid was, if anything, reduced (Sréter 1963). In our experiments, however, the total water was unchanged and the reduction of intracellular water was not significant.

It is known that muscular activity increases the ion exchange across the cell membrane (Hodgkin and Horowicz 1959). Loss of muscle potassium as a result of muscular activity has been found both in isolated muscle and in intact animals (Fenn 1936, Miller and Darrow 1941). On muscular contraction an increased potassium content in venous plasma from the group of muscles in question has been demonstrated in man (Farber *et al.* 1951, Hultman and Bergström 1962, Bergström and Hultman 1966 a). It has been suggested that lack of potassium might be a limiting factor on the contractility of the muscle. Miller and Darrow (1941) showed in rats, however, that the physical working capacity was not definitely diminished even under conditions of severe potassium depletion.

Deposition of potassium together with glycogen in liver tissue has been reported by Fenn (1939) and others. A similar relation between potassium and glycogen was found in isolated diaphragm (Calkins, Taylor and Hastings 1954). Nichol (1958), on the other hand, found no relation between muscle potassium and glycogen in man (biopsy specimens taken in connection with operations).

In our experiments potassium in the extracellular fluid (plasma) increased in all cases. The concentration rose immediately after the start of work, thereafter increased slightly and fell back again immediately after the end of work. In muscle tissue potassium fell in relation to GFFS indicating that potassium had left the working muscle together with glycogen. The very slight decrease of potassium (about 4 per cent of the basal value) cannot have been a limiting factor on the physical working capacity in these experiments.

During the exercise there was an increase of plasma phosphorus while the phosphate excretion in the urine was not consistently changed. The increase of plasma phosphorus indicates a loss of phosphorus from the working muscles. No significant lowering of phosphorus however was recorded in muscle, perhaps due to the slight reduction in relation to total phosphorus.

To summarize the most pronounced change in parameters measured during prolonged physical exercise is the decrease in local glycogen store in working muscles. It is therefore suggested that this glycogen store is a determinant for the performance capacity in this type of work.

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Effect of Prolonged, Heavy Exercise on Urinary Protein Excretion and Plasma Renin Activity

By

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Abstract

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Experimentally induced proteinuria by renin injection is a well-known phenomenon. The aim of this investigation is to study the effect of prolonged, heavy muscular exercise on plasma renin activity and its possible connection with exercise proteinuria. In a group of 13 male participants in the Vasaloppet in 1965 (85 km ski race) the urinary protein excretion was studied before and during the ski race, and the plasma renin activity at the start and the end of the race. The increase both in plasma renin activity and in urinary protein excretion was statistically significant. During rest, before the ski race there was a significant correlation between plasma renin activity and protein excretion in the urine (r = 0.84) but not during the ski race.

The reversible physiological increase in protein excretion in urine during exercise is well-known. The physico-chemical nature of the urinary proteins during and after exercise has been studied by several authors (Nedbal and Seliger 1958, Rowe and Soothill 1961, Poortmans, van Kerchove and Jaumain 1962). It is the plasma proteins, mainly the serum albumin, whose urinary excretion is increased during muscular exercise. The view most generally accepted is that proteinuria is caused chiefly by increased glomerular permeability (Poortmans *et al.* 1962, Zaruba and Fixa 1964).

An increased protein excretion in urine has been produced experimentally by injection of renin, probably due to an increase in glomerular permeability (Seiler *et al.* 1932). After induction of renin proteinuria in rats electron microscopy has shown glomerular changes which are considered to be related to increased permeability of the glomerular capillaries (Fisher and Masson 1961). The effect of renin may be a direct effect on the glomerular permeability for large *molecules* and/or secondary to the vasoconstrictor effect of angiotensin. Very little is known about the effect of muscular exercise on the renin angiotensin system. The results obtained by Helmer (1964) and our own studies of renin release during short muscular exercise on a bicycle ergometer (Božović and Castenfors, 1967) indicate that plasma renin

activity is increased during muscular exercise The aim of the present study was to test if prolonged, heavy muscular exercise also causes an increase in plasma renin activity and if a correlation exists between this activity and the urinary excretion of protein. This study forms a part of a more detailed investigation on renal function during prolonged, heavy exercise (Castenfors, Mossfeldt and Piscator 1967)

Material and methods

"Vasaloppet" is an 85 km ski race which takes place once a year in Sweden. The test group consisted of I mal participants in this race. Their ages varied between 24 and 63 The skiing time was between 6—10 hrs. For further details see Castenfors, Mossfeldt and Piscator (1967)

Urine was collected on tw different occasions, the first sampl was taken in the morning before the race and the second t the end of the race. The urine was kept deep frozen until the analyses was made. A biuret micro method according to Goa (1953) was used for determining the urinary protein. For further details see Piscator (1962) Creatinine was determined with an autoanalyser according to Hawk, Oser and Summerson (1947) Samples of bout 25 ml enous blood were taken before the start and t the end of the race. These were centrifuged t room temperature, and the plasma was deepfrozen. The plasma renin activity was determined according to Boucher *et al.* (1964) with the modification that heparin was used as anticoagulant instead of EDTA. Current statistical methods were used.

Results

Table I shows the plasma renin activity and the urinary protein excretion.

There was a statistically significant ($P<0.02$) increase of plasma renin activity at the end of the race compared with the plasma level at its start, although there were

TABLE I The effect of prolonged muscular exercise on urinary excretion and plasma renin activity

	Urinary protein excretion mg/g creatinine			Plasma renin activity ng angiotensin/100 ml plasma		
Subject	Before A	During B	Difference B—A	Before A	During B	Difference B—A
1	36	250	+214	260	1 610	+[illegible]
	9	75	+ 66	—	900	
3	74	96	+ 22	330	2 188	+1 858
4	48	203	+160	120	—	—
5	—	91	—	90	[illegible]	+ 60
6	41	285	+244	110	250	+ 140
7	60	88	+ 28	180	800	+ 620
8	113	125	+ 12	510	490	— 20
9	44	168	+124	80	610	+ 530
10	53	81	+ 26	70	330	+ 260
11	49	0	+ 21	120	300	+ 180
12	60	232	+172	10	230	+ 20
13	35	175	+140	70	130	— 60
14	38	192	+[illegible]	60	920	+ [illegible]
15	[illegible]	228	+1 0	110	170	+ 59
mean	31	[illegible]	+111	160	639	+ 471
SE	6	19		34	170	
P			<0.01			0.02—0.01

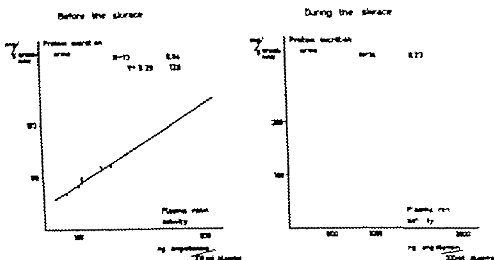


Fig. 1. Correlation between plasma renin activity and protein excretion in urine, before and during the 1965 ski race.

wide individual variations. A statistically significant increase ($P < 0.01$) in the urinary proteins during the race was also observed.

The correlation between the plasma renin activity and the urinary protein excretion is shown in Fig. 1.

During rest, before the race, there was a statistically significant, positive correlation between the protein excretion and the plasma renin activity ($r = 0.84$). The protein excretion during the race showed no significant correlation with the plasma renin activity ($r = -0.23$).

Discussion

An interesting finding in this study was the substantial increase in the plasma renin activity in connection with the ski race. The highest values are comparable with the increased renin values in some patients with ascites or renal artery stenosis (Genest *et al.* 1964). The physiological significance and the factors causing this substantial increase in the release of renin from the kidney during heavy exercise are not known. There is convincing evidence that renin is secreted by the renal juxtaglomerular cells, but the nature of the signal for release perceived by the cells remains obscure. A recent review of the renin angiotensin system has been published by Peart (1965). There are two main theories, 1. the renal afferent glomerular artery acts as a baroreceptor, 2. the macula densa responds to changes in the composition (sodium or osmolarity) of the distal renal tubular fluid. Both factors might contribute to the increase in plasma renin activity during work. The catecholamine induced renal vasoconstriction may act on intrarenal baroreceptors and the loss of sodium and water owing to perspiration may change the renal tubular handling of sodium, and stimulate renin release. The renin angiotensin system has been regarded as participat-

ing in the regulation of the plasma volume, and it is possible that its decrease during exercise aggravated by dehydration during such prolonged, severe exertion may be the stimulus for the increased renin release

The increased protein excretion is in accordance with earlier studies on exercise proteinuria. The high correlation (r = 0 84)during rest between plasma renin activity and urinary protein excretion is surprising, when taking into consideration the long period for collecting urine and the fact that there was only one plasma sample at the end of the period. This may indicate that renin plays a role in the variation of physiological proteinuria. During exercise this *correlation disappears, and even a* negative correlation tends to occur This may be due to the rather pronounced changes in renal hemodynamics which are observed during exercise and which contribute to the production of the proteinuria. It is also possible that the urinary protein excretion is, throughout the race, more directly related to the plasma renin activity during the race, than at its end In this test group the wide individual variations in exercise proteinuria seem to a great extent to be dependent upon a constitutional factor probably located in the glomerular membrane (Castenfors, Mossfeldt and Piscator 1967) to which may also be ascribed the absence of a correlation between plasma renin activity and urinary protein excretion.

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In vitro Assay of the Effect on Bone Marrow Cell Proliferation of Factors in Serum from the Irradiated Rat

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Abstract

CEDERBERG A., E. NIKKANEN and T. RYTÖMAA. *In vitro assay f the effect on bone marrow ell proliferation f factors in serum from the irradiated at* Acta physiol scand. 1967 *70* 147—157

An ttempt as made to demonstrate the existence or non-existence in rat serum of humoral factors which could be held accountable for the true regeneration process in the hole body irradiation-damaged bone marrow cells. The effects of the test serum, ollected at the time of onset of true regeneration (10 days after whole body irradiation of 600 r) were assayed *in vitro*, using normal bone marrow ells as target tissue. The results demonstrated that, on the average, the proportion of H-thymidine labelled cells was bout 24 per cent higher in cultures incubated in the presence of test sera than in the controls.
On the basis of partial differential ounts (per cent mature granulocytes) and measurements of total radioactivities incorporated in the hole population of ells (both estimations being performed on some of the preparations also analysed by autoradiography) it as concluded that the effect of the WBI serum as due one or more factors hich convert esting progenitor cells to the generatin cycle

It ha long been known that extensiv cell damag occurs in the haematopoietic tissues after massive whole body rradiation WBI Injury becomes visible in the bone marrow shortly after irradiation. extensive depopulation, intramarrow hemorrhages nd certain structural changes are manifested during the first day After 700 whole body rradiation, t has been observed n the rat that nucleated bone marrow ells ar t minimum 3 to 4 day after WBI (see e.g Cederberg 1965 Between da 4 and 6 signs of focal proliferation can be seen in the bone marrow primaril n ol ng granulocyt prog nitor cells This stage is often called the abortiv gr ulocytic rise because t is followed by a new drop in cell numbers True eg neration n at bone marrow is not seen until 8—9 days after WBI with 700 cell prolifer t n nc longer occurs n solitary foci but rather diffusely throughout the marrow space Cederberg 1965

It is clear that the marked changes in the bone marrow which follow WBI are also reflected in the peripheral blood. The changes in the blood picture are not parallel with events in the bone marrow however either from the quantitative and qualitative or from the temporal point of view In view of certain specific inferences presented later in this article, it may be worth recalling that in the blood the leucocyte count begins to rise 9—10 days after WBI and in the tissues even later The erythrocytes, on the other hand, continue to decrease even at this time on the 9th day the values recorded were less than 20 % of the original level (Cederberg 1965)

Dynamics of the reparative processes in the bone marrow are not fully understood at present. It has been suggested, however that certain physical factors, such as the decreasing pressure in the bone marrow cavity which follows resorption of the haemorrhage and cell debris, plays a regulatory role in the repopulation process after WBI (Lamerton et al. 1959) The suggestion is plausible, but such factors should evidently be distinguished from the specific r gulatory substances which control mitotic activity in adult mammals (Bullough and Rytomaa 1965) The role of growth pressure" hovever might be more important under the unique circumstances following WBI than in the numerous other instances in which apparently similar regeneration takes place in the bone marrow Whatever the true situation turns out to b., t may at present be wiser to consider growth pressure not as the cause of regeneration but rather as a necessary environmental condition.

In the present study an attempt was made to demonstrate the existence or non-existence of humoral substances which could be held accountable for the true regeneration process in the bone marrow cells without the concomitant action of such (secondary) factors as "growth pressure In short, the presence/absence of substances in the serum of irradiated rats at the time of onset of true regeneration (10 days after WBI) was tested in vitro using normal bone marrow cells as target tissue.

Material and methods

The est animals comprised 20 whit male rats of Sprague-Dawley strain (bred t Medica Orion Pharmaceutical Company Finland) Their ages ranged from 6 t 8 months, and eights from 200 to 300 g The animals had free access to food and water

Eigh rats were exposed to whole body irradiation (WBI) ventilated plexi-t bes, one rat in each tube placed radially on wooden stand. The dose administered was 600 under the following physical conditions irradiation w th ^{60}Co, TSD 143.5 cm, nd dose-rat 22 rads minute I previous study from our laboratory in which animals of the same colony were exposed WBI with 700 (Cederberg 1965) it was observed tha some of he rats died before the onset of the true repopulation process in the bone marrow and ha the survivors often showed signs of bacterial infection, presumably of oral and in estinal origin (Quastler 1956, Cederberg 1965) As both these events were undesirable for the present purpose the radiation dose administered was reduced to 600 r It was thought tha further reduction might crea temporal complications in he desired WBI syndrome None of the rats exposed 600 died before the me of kill ng (10 days after WBI) nor were they particularly sick

Test sera were collected from the irradiated rats 10 days af er WBI control sera were collected at the same time from the control animals Blood was withdraw from he rats under light ether anaesthesia by puncture of the vena cava inferior and the serum was separa ed and stored — 20 C until used for experiments. Eigh est sera and an equal number of controls were assayed the experiments reported here; the total number of cultures analysed as 92

The bioassay method adopted here was closely similar t the techniques used in earlier studies (Rytömaa and Kiviniemi 1967) Normal bone marrow cells, used as the target organ in th tests, ere obtained from adult male rats belonging to the same colony of animals as the serum donors. The cells were suspended in Hanks' balanced salt solution (BSS) and an equal number of cells was added to each culture of the experiment, adjusted to yield 500—1000 nucleated cells per mm² in the final medium. The total olume of each cultur was 0.4 ml.

The culture medium onsisted of Hanks BSS as basal solution and 20 % of serum (test or control, espectively) ³H-thymidine (³H TdR) specific activity 3 C/mM (The Radiochemical Centre, Amersham, England) was used as label It was added to the cultures in concentrations ranging from 0.25 to 0.50 μc/ml, as indicated in the results.

Individual cultures were prepared according to monolayer technique as described by Mer hant *et al* (1960) I this system the cells settle from the suspension nd become attached to the upper surface of coverslip (10 × 20 mm) hanging from the top of Pyrex-glass tissue cultur tube (Ø 12 mm) All tubes were tightly stoppered and incubated in horizontal position at 37° C for 6—24 hrs. Air was always used as the gas phase in the cultures. It goes ithout saying that aseptic techniques nd other generally accepted principles to tissue culture work were also followed in these short-term incubations.

After the desired incubation time the coverslips were harvested, the excess of medium drained off, and the preparations allowed to dry in air The cells were fixed *in situ* in 96 % ethanol, washed in distilled water and dried. The preparations were then mounted face up on microscope slides after his, toradiograms were prepared by the Kodak AR-10 stripping film technique After exposur room temperature in the presence of small mount of silica gel, the utoradiograms ere processed according to the instructions given by Kodak. The cells were stained with Mayer' haematoxyli or dilute Giemsa solution. Code-numbered autoradiograms ere analysed by determining the proportion of labelled cells this was achieved by counting the number of cells until 100 labelled cells (> 10 grains) had been found in each preparation. As the background "labelling" constantly remained at or below 1 grain per 100 μ^2 hence labelling could not affect he results. On the other hand, truly labelled ells containing less than 10 nuclear grains er rare in the preparations analysed, and ell ithin the statistical counting error

I one experiment (IV) the preparations ere first counted for total radioactivity incorporated in he cells by measuring the activities in liquid scintillation counter (Isotope Developments Ltd., England) Th technique adopted was as described earlier (Rytömaa nd Kiviniemi 1967) After hese measurements of total radioactivity each preparation was washed in series of solutions of dioxan nd ter to remov all scintillator liquid from the samples. The preparations wer then treated by the the technique described bov to obtain utoradiograms.

Partial differential counts were made on some of the preparations This as done by determining the relative mber of mature granulocytes from code-numbered preparations.

Statistical significances of differences bet een the groups er primarily estimated by means of Wilcoxon non-parametric method" (rank test) Further statistical analyses using pooled data and larger groups of measurements, ere performed by means of Student's t-test and analysis of ariance (Simpson *et al* 1960)

Results

In the first experiment the cultures were incubated for 6 and 24 hrs in the presence of 0 25 μc ml of ³H-thymidine Autoradiographic analysis of cod numbered preparations revealed that the proportions of labelled cells were on the average 38 and 14 per cent higher in cultures containing serum of irradiated rats than in controls at 6 and 24 hours espectively Tabl I) At 6 hrs the difference was statistically signifi cant at the level P = 2 α ≈ 0 02 according to Wilcoxon's rank test Although the mean labelling index was higher in the WBI group even at 24 hrs the difference between this group and the controls was not tatistically significant

In the second experiment ³H TdR was used in a concentration of 0.35 μc ml the incubation t mes in this case wer 6 and 18 hrs At six hours the control cultures conta ned 10 6 0 6 mean S E M per ent labelled cells and at 18 hrs 13 6 ± 0 7 The corresponding figu es n the test group containing of serum of rats 10 days after WBI were 13 1 0 7 and 16 6 ± 0 9 per cent, respecti ely (Fig 1)

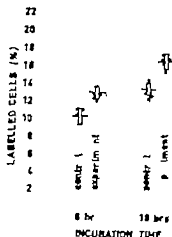


Fig 1 Per cent of labelled normal bone marrow cells incubated *in vitro* in the presence of test nd control sera. *In the figure both the individual alues and the corresponding means* (± S.E.M) re shown.

According to Wilcoxon s non-parametric test the two-sided significances for the differences between the groups were P ≈ 0 02 at 6 hrs, and P ≈ 0 01 at 18 hrs. Analysis of variance from data in which the subgroups were equalized regarding the number of observations indicated that the serum effect (F = 16 5) was significant at the level P < 0 01 and that the "incubation time effect (F = 21 4) was also significant at the level P < 0 01 There was no detectable interaction between these two main effects, however (F < 1 0) The biological interpr tation of the latter finding is that the sera did not modify the time effect" or *vice versa*, which is consistent with the previous findings (Rytomaa and Kiviniemi 1967) that similar serum factors do not alter the rate of division per labelled cell

The third experiment was a formal replica of the second, except for the staining of the autoradiograms. The proportion of labelled cells was again higher in the group containing WBI serum than in the controls, both at 6 and at 18 hours (Table 1) On the basis of the information given by the two previous experiments, estimation of the statistical significances between the group differences could now be based on one-sided tests. According to Wilcoxon s rank method, the significa ces were P ≃ 0 005 and P ≃ 0 01 at 6 and 18 hrs, respectively

In the fourth experiment H TdR was added to the cultures in a concentration of 0 5 μc/ml After incubation times of 6 and 18 hrs the cultures wer harvested and prepared for liquid scint llation counting to measure the total incorporation in terms of absolute radioactivity per unit population of bon marrow cells Owing to the relatively low counts recorded in 100 sec per preparation ach of them wa counted twice, and the results pooled This reduced the average counting error (sample + background) to less than 3 per cent wh ch wa now insignificant in comparison to the variation between samples The results ndicated I g 2 that WBI serum had higher total radioactivities than the controls the group differences were significant at the level P ≈ 0 02 at both 6 and 18 hrs.

Two highest and two lowest alues of the 18-hr groups ere substit ted w h he respectn means As consequence degrees of freedom of the whole ma erial ere reduced by 4

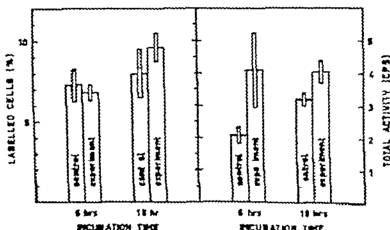


Fig. 2. Labelling of normal bone marrow cells *in vitro* in the presence of test and control sera. I the right part of the figure are show total radioactivities incorporated in the hole population of ells (counts per second; mean ± S.E.M.) in the left part are seen the relativ umber of labelled cells in the same preparations (mean ± S.E.M.)

After measuring the total radioactivities, the same preparations were processed to obtain autoradiograms. F om these relative labelling was determined a usual the results are seen n Fig. 2 and Table I The first 'failure was observed in these analyses at 6 hrs no difference was found between cultures incubated in the presence of WBI serum and of normal serum At 18 hrs however a significant difference was again observed (P ≈ 0.005) It may deserve emphasis that the large standard error of the control mean at 18 hrs (Fig 2 and Table I) is due to one preparation which deviated ery strongly f om the others of the group Although this single value could have been rejected from the material according to adequate statistical criteria (the alue wa too larg with $P < 0.005$ this wa not done because the deviation was obviously due to hance error in the autoradiographic analysis, as judged from the fact that liquid scintillation count ng did not eveal such a difference Further more ow ng to the elati ely light i fluence in W lcoxon rank test, of a single deviation no matter how large this erroneous alue failed to mask the statistical significanc of the differen betw en th two groups

So that all autoradiogr phic measurement of the four different experiments could be pooled the raw data wer t ansformed to standardized index figures. This was achieved by div ng the ndividual per cent positivities n each group by the appropriat ont of median The median was ndered mor su table for thi pur pose than the mean bec use t les affected by such ext eme deviations a that detected n expt IV Di tribution diag ms of the alculated ndices demonstrated that for all pra tic l pu pose I t h ndividual lues in the ont ol group wer normall distribu d ound the me whereas the f equency diagram of the expe mental ndices expe ted I t l kewed to the right The mean and their

TABLE I Relative number of labelled cells after incubation of normal bone marrow cells ... represent labelling of the cells in relation to the appropriate control medians (= 100)

Number of experiment		I		II	
Incubation time (hrs)		6	24	6	18
Relative number of labelled cells Mean ± S.E.M. (Median contr. = 100)	Control serum	115±27	98±9	97±7	101±4
	WBI serum	162±16	116±9	119±5	129±8
Increase (per cent of control)		+38	+38	+14	+21

standard errors are given in Table I. It is seen from these values that on the average the relative proportion of labelled cells was about 24 per cent higher in cultures incubated in the presence of 10th day WBI serum than in the controls. According to Student's t test, this difference is highly significant ($P < 0.001$).

The whole material studied consists of four independent blocks of measurements regarding cell labelling with ³H-thymidine and autoradiographic analysis. The measurements have been influenced not only by differences in the serum types, but also by different environmental conditions which were deliberately varied between the blocks. These include differences between the animals (16 serum donors and 4 cell donors) as well as between the assay conditions. Among the latter are such factors as concentration of the label, cell number and autoradiographic procedure. It is thus apparent that the joint action of the different environmental conditions must be unpredictable and unique for each block of measurements. Consequently generalized conclusions as to the effect of the serum alone must be based on its action as distinct from that of the other effective factors.

For this reason the data were subjected to analysis of variance, the types of sera and the different environmental conditions being considered as the two sources of variation. Due to computational difficulties, corrected mean values of the different subgroups were used in the analysis. Correction of the means was achieved by transforming all values to the level observed at 18 hrs.

It was suggested on theoretical grounds and in view of previous knowledge (Rytömaa and Kiviniemi 1967 and unpublished) that the relative number of labelled cells probably increases linearly as a function of incubation time. In accordance with this, the linear correlation between the 6-hour and 18-hour values was very high ($r = +0.92$ in this study also), thus indicating that the "least squares" regression equation, applied for the correction of the data, could not alter the results to any significant degree.

Analysis of variance demonstrated that the level of cell labelling was significantly affected both by the type of the serum and by the environmental conditions ($P < 0.01$ with $F = 40.0$ and $F = 84.0$, respectively). However, interaction of these two effects did not appear significant, which is interpreted to mean that interdependence of the

There were unequal numbers of measurements within the different subgroups, which was partly due to uncontrollable circumstances, such as accidental culture fail

in the presence of control and test sera. The index figures (mean ± S.E.M.) shown in the table

III		IV		Grand mean
6	18	6	18	
113 ± 20	106 ± 9	101 ± 14	116 ± 21	106
169 ± 16	150 ± 11	95 ± 6	138 ± 6	131
+47	+42	−6	+19	+23.6

TABLE II. Analysis of variance using indices obtained in five independent blocks of measurements. Note that the analysis is based on the relativ effects of the different serum types.

Source of variation	Sum of squares	Degrees of freedom	Mean squar	F	Significance P
Effect of treatment (serum type)	A 6 035	1	6 035	12.07	< 0.01
Effect of experiment (serum batch)	B 1 707	4	426.8	0.85	insignificant
Effect of AB-interaction	AB 1 702	4	425.5	0.84	insignificant
Random deviations	5 045	10	504 5		
Total	14 509	19			

main factors was either weak or nonexistent. Essentially the same result was also obtained by analysis of variance based on the assumption that the serum effect, *e* stimulation, was proportional to the level of the corresponding control value. It may be mentioned that n this analysis, based on logarithms of the values the interaction effect was still further reduced

On the basis of assumptions of insignificant ntera tion ffects and of the relativeness of the action of the serum factors, the data were also tested in a different way. The analysis was performed with the ndex figures shown in Table I supplemented with similar indices calculated f om the total adioactivities. Although the result of the analysis wa essentially unaffected even if the last mentioned indices were excluded, there is good eason t consider them as independent data. This conclusion is based on the fact that absence of significant nteraction between envi onmental conditions and t pe of serum ha made the ctual dependenc rrelevant to the problem.

The esults of the anal sis are seen n T ble II

For the sake of clarity t is emphasized that the wa   n which the different indi es wer calculated failed t   ause ppreciable hanges in the random deviations as ompared with those seen befor

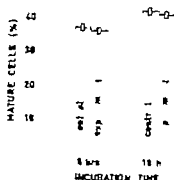


Fig. 3. Per cent of mature granulocytes (mean $\pm$ S.E.M.) in cultures of normal bone marrow cells incubated in the presence of test and control sera. Not that the increase in time is statistically significant ($P < 0.01$ according t analysis of variance)

In order to detect any gross population shift among the bone marrow cells incubated in the presence of each of the two different serum types, some partial differential counts were made. These were performed on the code-numbered preparations of expts. II and III by counting the number of mature granulocytes per 100 nucleated marrow cells. The results are illustrated in Fig. 3. Analysis of variance showed that the slight decrease which occurred between the test and control groups is entirely insignificant and must be considered the result of pure chance. This is quite clear from the F value obtained which was less than unity thus indicating that the group means vary less than expected from the individual counts. The accidental nature of this difference is further strengthened by the insignificant interaction effect in this case, too the F value was less than unity. On the other hand, the effect of the second main variable, time, was significant ($P < 0.01$)

Discussion

Detailed knowledge of the specific mechanisms that control the rate of blood cell production is at present meagre. Until recently only one substance belonging to this category has been firmly established erythropoietin (see Jacobson and Doyle 1962). Much work has been done with the purpose of demonstrating the existence of other haemopoietins but the result has usually been new contradictory findings and puzzling hypotheses. In the opinion of the present authors, the situation is mainly due to the joint action of two factors the complex physiology of blood cells and the lack of unambiguous assay methods.

It has been clearly stated by some authors that certain clinical and experimental observations indicate the existence of a dual control mechanism for red cell production (Brecher and Stohlman 1959. If analogous reasoning — and reasons — are applied to the other blood cells, the existence of at least 6 specific regulator substances must be inferred to some extent this arises from the mitotic independence of the various blood cell systems. As a matter of fact, data are accumulating which indicate that all mitotic control mechanisms have certain common themes and that the

various mechanisms found in different cell systems are merely variations on these themes (Bullough and Rytömaa 1965)

At least three specific mechanisms have been suggested to control blood cell production poietins, chalones, and (granulocytic) "antichalone" (Rytömaa and Kiviniemi 1967) There is good experimental evidence for supposing that erythropoietin acts at the level of pluripotential (Ford *et al.* 1956 Becker *et al.* 1963 Duplan 1967) stem cells, inducing them to differentiate into the erythrocytic system (Lajtha *et al.* 1964 Lajtha 1967) Granulopoietin, if considered as the formal analogue of erythropoiet n, would have a fundamentally similar mode of action. Despite all this, it is clear that the total flux from a stem cell compartment to granulocytic and erythrocytic system is quantitatively very small (Lajtha *et al.* 1964 Patt and Maloney 1964 Cronkite and Fliedner 1964) An important consequence thus emerges that any realistic poietin effect is necessarily difficult, if not impossibl to detect by assay methods involving DNA labelling in short term incubations of normal bone marrow cells. As a matter of fact, this inference has been experimentally confirmed both in our laboratory (Rytömaa and Kiviniemi 1967) and elsewhere (Alpen 1962, Erslev 1965) Therefore, the results observed in this study are not explicable in terms of poietin action.

It has been demonstrated by recent partly unpublished observations (Rytömaa and Kiviniemi 1967) that granulocytic and erythrocytic chalones, which act as mitotic inhibitors, seem to originate f om mature circulating cells. It has been possible to extract thes. chalones (together) from the particle-free medium in which intact blood cells hav been incubated for short periods of time as w ll as from certain types of blood serum. These findings are in full agreement with those reported earlier by Craddock (1960 1962)

The mode of action of bone marrow chalones" appears to be two-fold they prevent progenitor cells from entering the generative cycle and, in addition slow down certain processes n cells that ha e already embarked on cy le Thus, chalones seem to prolong the duration of the DNA synthesis phase as judged from the reduced grain counts in autoradiography It may require emphasis in this context that purified chalone fractions do not contain nucleotides or other competitors for thymidine which would dilute the ncorporation of H TdR, as judged from the UV absorption pectra Furthermore the halone ffect has been demonstrated equally successfully w th other labelled precursors besides th midine.

Granulocytic antichalone has been d monstrated in certain types of rat sera and their fractions This factor has been found to onvert only myelocytes f om d chophase G_0 t generativ cycle This point of action is the main reason why antichalone and granulopoietin are considered to be different factors

It is known from an earl er study Cederberg 1965 that 10 days after 600 r WBI true egeneration is n process in the bone marrow in the colony of ats used her It w further ndicated by the results f th study cited that at the same time there a trong demand for gr nulocytes n the periphery (defence barrier The situation is obviously analogous to that existing after repeated leucapheresis, in which

granulocytic antichalone has been found in serum. Furthermore under both circumstances the absolute number of granulocytes in the whole body is evidently much below the normal level Thus, in turn must mean that the concentration of granulocytic chalone in the organism is subnormal

The same arguments apply to erythrocytic chalone, too. Ten days after WBI the rats are gravely anaemic according to the values reported (Cederberg 1965) the red cell count is hardly more than 20 per cent of the original. One would thus obviously expect elevation of granulocytic antichalone, and simultaneous reduction of both chalones in the WBI sera tested here. Furthermore the modes of action of the test factors suggested that the response would primarily be due to an increase in the number of labelled cells.

This was exactly the result obtained in this study Not only did the observations agree qualitatively with expectation, but strikingly well even quantitatively It may be recalled that in the earlier studies (Rytömaa and Kiviniemi 1967) the mean TdR incorporation was numerically 4 8 % per cent higher in the absence of all chalones than in the controls, although this difference was not statistically significant. Because the stimulation caused by granulocytic antichalone in turn, was 19 6 per cent one would expect that with an assay material such as was used here the overall stimulation would possibly be little more than 20 per cent As shown in Table I the observed overall stimulation was 23 6 per cent

Despite the exact agreement between the numerical stimulation observed and predicted, it is not possible that the reaction intensities could have been determined by the test substances alone, particularly at the level observed in this study After a given limit, the responsiveness of the cell population itself becomes the most important factor Normal bone marrow cells show such a high mitotic activity that an instantaneous increment in this figure, exceeding some 25 to 30 per cent seems highly unlikely as far as the effect is due to triggering of resting progenitor cells in the generative cycle Much higher values would obviously suggest alteration of cell cycle phases, or the presence of some artefact. This, like some additional criticism which has been ignored in the present discussion, indicates that only the following conclusions are sufficiently firm to be regarded as established 1) At the time of true regeneration in WBI-damaged bone marrow rat serum contains one or more factors which convert resting progenitor cells to the generative cell cycle 2) The reactions observed are in good agreement with earlier more specific findings and theories.

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Excretion of Sodium and Potassium in Cat Submandibular Saliva

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Abstract

Petersen O H. and J H Poulsen. *Excretion of sodium and potassium in cat submandibular saliva.* Acta physiol scand 1967 *70* 158—167

The concentrations of ions in the saliva vary with the secretory rate Different results concerning sodium concentration in relation t flow rat have been found by different investigators. I the present work secretion was elicited by chorda stimulation and he concentrations of sodium and potassium in the saliva were determined by flame photometry The sodium concentrations in the first samples of cat submandibular saliva taken from previously resting gland were higher than the sodium concentrations in samples taken later t the same secretory rate The maximal difference in concentration between transient and steady state sample was about 50 meq/l. Steady stat concentrations of sodium increased proportionally t the flow rat except at low flow rates here sodium concentrations were greater than expected on the basis of this relation Steady state concentrations of potassium were independent of the secretory rate when this was higher han 100 µl/min/g gland, with a value of bout 10 meq/l. Below this flow rat the potassium concentra ion rose first slightly and then steeply when flow rate decreased, nd reached values of more than 100 meq/l at secretory rates of bout 2 µl/min/g gland.

The secretion of sodium and potassium in sali a has been the subject of a great number of investigations. In most glands t ha been demonstrated that the concentration of sodium and potassium in the saliva aries with the secretary rate (Thaysen 1960) Until recentl however the interpretation of the results has been very difficult because the differentiated functions of the acini and the different section of the ducts have been unknown

Thaysen, Thorn and Schwartz 1954) proposed that the acina primary secretion had a plasmalike composition and was modified n the duct sy tem by eabsorption of sodium chloride followed only by a small amount of water lea ing th final saliva hypotonic. This hypothesis ha been the ubject of much criticism (B rgen and Seeman 1958 Burgen, Terroux and Gonder 1959 and Burgen and Terroux 1963 Recently the truth of Thaysen, Thorn and Schwartz s hypothesis has been very much ubstantiated by micropuncture studies in rat salivary glands (Martinez, Holzgreve nd Frick 1966, Young and Schögel 1966 and Mangos, Braun and Hamman 1966)

in which the composition of the primary secretion was directly determined with respect to the concentrations of sodium, chloride and potassium. It was shown that the concentrations of these ions were nearly the same as the concentrations in plasma and that the composition of the primary secretion was independent of the secretory rate. It is therefore now possible from the concentrations of electrolytes in the final saliva to calculate the amount and direction of the net electrolyte transport in the duct system, if one assumes that the acini are the only place where net secretion of water occurs (Young and Schögel 1966).

In such calculations it is very important to know whether the measured concentrations are "transients" or represent a steady state. Burgen (1956 a) described transient high potassium concentrations in the first samples of parotid and submandibular saliva secreted by a previously resting gland. In the present work a similar but more protracted "sodium transient" has been demonstrated. When precautions to prevent "sodium transient" were taken, it was possible to obtain steady state curves for sodium concentration in saliva versus secretory rate with a very small spreading. Furthermore, the steady state concentration of potassium in the saliva has been investigated, and it has been shown that the potassium concentration which at high flow rates is comparatively low in the cat submandibular saliva, can reach intracellular levels at very low secretory rates.

A preliminary report of this study has been given (Petersen and Poulsen 1966 a).

Methods

Cats weighing 2—4 kg were anesthetized with chloralose (70—90 mg/kg intraperitoneally). The submandibular glands and their ducts were prepared as described by Liddell and Sherrington (1929). Salivary secretion was elicited by electrical stimulation of the parasympathetic secretory fibres (the chorda tympani fibres of the lingual nerve, which was divided centrally to the stimulating electrodes. During the experiment the nerve was entirely covered by paraffin oil. Stimulation was applied by a Grass stimulator using pulses of 10 msec duration, voltage of 10 V and frequencies from 0.1 to 25/sec. The saliva was collected from a polyethylene cannula in the submandibular duct into a tuberculine syringe.

In the transient experiments the collection of saliva began immediately after the start of the secretion. The first ... collecting periods were of half minute duration each, the following periods lasted one minute each.

To obtain steady state values the following precautions were taken. Salivary secretion was started at low frequency and the gland was allowed to secrete for 30 min before the first sample was taken. *Secretory rate was increased by increasing the frequency in small steps. After each shift* of frequency the gland was allowed to secrete in 10 min before a salivary sample was taken.

Salivary samples were always of the order of 20—300 µl. A Baird Atomic flame photometer with lithium as an internal standard was used for the determinations of the sodium and potassium concentrations. The accuracy of the determinations was checked in the following experiment. Aliquots of solutions containing 120 meq/l sodium chloride and 9 meq/l potassium chloride respectively were delivered into 10 weighing bottles and thereafter treated like salivary samples. The mean concentrations of sodium and potassium found by analyses were 122 ± 1.5 S.E.M. (standard error on the mean) and 9.0 ± 0.1 meq/l respectively. The possibility of an error in the determinations due to interference between sodium and potassium was investigated. The results showed that potassium concentration measured in the flame photometer was independent of sodium concentration in the range between 10 and 50 meq/l, likewise the sodium concentration measured in the flame photometer was independent of potassium concentration in the range between 10 and 100 meq/l.

When the experiments had been finished the glands were removed and weighed in order to be able to express the secretory rate as µl of saliva secreted per minute per g wet gland weight. The range of weights for submandibular glands in our experiments was 0.7—1.5 g.

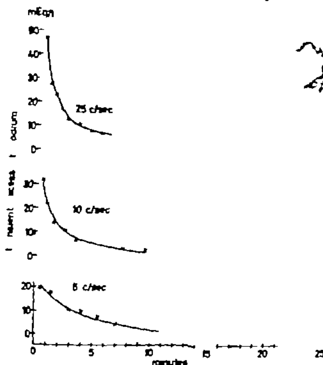


Fig 1 Effect of varying stimulation frequencies on the size of the "sodium transients" Transient excess of sodium concentration (the difference between the sodium concentrations in transient samples and the sodium concentrations in steady state samples collected at the same secretory rates) is shown as function of time after start of stimulation. Preceding resting period 1 hr Data obtained from one typical gland.

Results

Transient concentrations

In pilot experiments undertaken to investigate the relationship between the concentrations of sodium and potassium in the saliva and the secretory rate, care was taken to omit potassium transients by waiting 5 min after the start of stimulation before the first sample was taken (Burgen 1956 a) Nevertheless, the sodium concentrations in the first samples (stimulation frequency 10 c/sec) were significantly higher than those obtained later at the same secretory rate.

Dependence on frequency of stimulation

The relationship between the magnitude of these sodium transients and the stimulation frequency was then studied As the sodium concentration of the saliva varies with the secretory rate and as the secretory rate diminishes especially during the first minutes of stimulation, the sodium transients were expressed as the difference between the measured transient concentration and the steady state concentration at the same secretory rate As it was not possible to obtain steady state sodium concentrations at the highest secretory rates achieved in the first periods of some transient experiments, the steady state concentrations in these cases were obtained by extrapolation of the linear part of the curve representing sodium concentration versus flow rate (Fig 3) The dependence of the sodium transients on the stimulation frequency is shown in Fig 1 With increasing stimulation frequency the sodium transient increases. The highest values found in these experiments at a stimulation

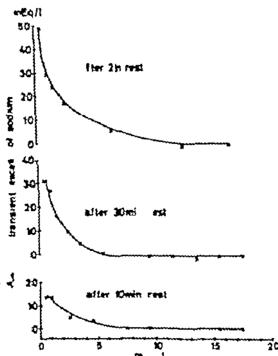


Fig. 2. Effect of varying durations of the preceding resting period on the size of the "sodium transients". Transient excess of sodium concentration is shown as function of time after start of stimulation. Stimulation frequency 10 /sec. Data obtained from one typical gland.

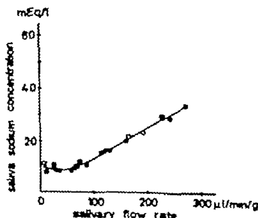


Fig. 3. Steady state sodium concentration as function of secretory rate. Data obtained from one typical gland.

frequency of 25 c/sec were about 50 meq/l in excess of the steady state concentrations; the sodium concentrations measured in the transient samples were in these cases about 120 meq/l.

Dependence on duration of the preceding resting period

The dependence of the "sodium transient" on the duration of the preceding resting period is shown in Fig. 2. With decreasing resting period the "sodium transients" decreased and nearly disappeared when the resting period was below 5 min.

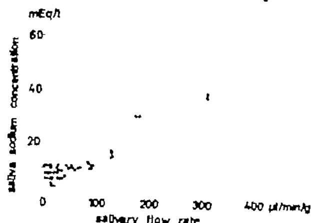


Fig. 4. Steady state sodium concentration as function of secretory rate. Data obtained only from glands having the same pattern of sodium excretion as the one shown in Fig. 3.

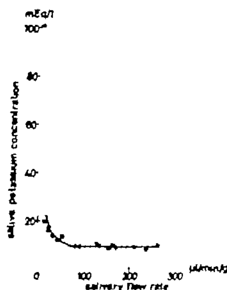


Fig. 3. Steady state potassium concentration as function of secretory rate. Data obtained from one typical gland.

Potassium transients were noticed in these experiments and showed the same properties as those described by Burgen (1956 a).

Steady state concentrations

A typical steady state curve for sodium is shown in Fig. 3. This course of the sodium curve was found in 12 out of 20 expts. In 3 expts. the same linear relationship between the concentration and the flow rate was established, but in these cases the slope of the curve was greater. In the remaining 3 expts. a similar great slope was seen, but at greater secretory rates the curve flattened somewhat out. The linear relationship was broken in 15 out of 20 expts. in the lower flow rate range where the sodium concentration was greater than expected from this relationship.

A typical potassium steady state curve is shown in Fig. 5. Potassium concentration was independent of secretory rate when this was higher than 100 µl/min/g gland with a value of about 10 meq/l. At secretory rates below 100 µl/min/g gland the potassium

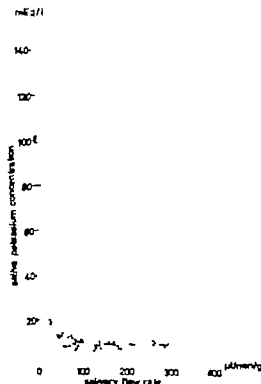


Fig. 6. Steady state potassium concentration as function of secretory rate. Data obtained only from glands where sufficiently low flow rates were reached.

concentration rose first slightly and then steeply when the flow rate decreased, and at flow rates about 2 µl/min/g gland values of about 100 meq/l were reached. These very high potassium concentrations have been measured in the saliva of 15 glands from 11 cats (all the experiments where sufficiently low secretory rates were reached).

The possibility that the very high potassium concentrations could be due to a large content of cells in the samples at the very low secretory rates could be excluded. If this was the true explanation the whole salivary samples at the lowest secretory rates should consist of cells. However, the salivary samples at these flow rates have just the same appearance as the samples taken at higher secretory rates. Furthermore, a control experiment was carried out to test this possibility. A salivary sample taken at a low secretory rate was centrifugated. The potassium concentration was measured in the upper layer and in the lower layer of the sample after shaking. No difference in the potassium concentration (40 meq/l) of the two layers was found.

It is interesting that in Fig. 4 and 6 showing sodium and potassium concentration as a function of the secretory rate the spreading for the sodium values seems to be somewhat greater than the spreading for the potassium values. This was also noticed by Thaysen (1935). In Fig. 3 it is however seen that this greater spreading in the sodium values is not due to a greater spreading for sodium in the individual gland, but the explanation is that the difference from gland to gland is greater with respect to sodium excretion than with respect to potassium excretion.

Discussion

As described in the introduction, a primary secretion with a plasma-like composition is formed in the acini. The ion transports involved in this fluid formation have not been entirely clarified yet, but Lundberg (1957 a, b, c) presented strong evidence in favour of the hypothesis that an active transport of chloride was the most important step. This was supported by Petersen and Poulsen (1966 b) who showed that acetazolamide which inhibits active chloride transport in a number of secretory organs (Davson 1964) is able to inhibit both the secretory rate and the secretory potentials in the acini.

In the ducts the primary secretion is made hypotonic by a reabsorption of ions without a corresponding subsequent water transport. Young and Schögel (1966) showed that in the rat submandibular gland this electrolyte reabsorption took place in the sublobular ducts which consist of striated ducts and granulated tubules. As the granulated tubules do not exist in the submandibular gland of the cat (Burgen and Emmelin 1961) it is most likely that the striated ducts are the place where the electrolyte reabsorption takes place as was originally proposed by Thaysen (1935). As this mechanism is sensitive to ouabaine (Mangos and Braun 1966) and the potential difference across the duct wall is so orientated that the lumen is negative (Lundberg 1955) it is probably sodium that is the ion actively transported. It is likely that part of this outward transport of sodium is linked with the inward potassium transport which takes place in the duct system.

Steady state concentrations

Sodium

Our results concerning the steady state excretion of sodium in saliva, as presented in Fig. 3 and 4 are easily explicable on the basis of this model and are in accordance with previous results obtained by among other Thaysen, Thorn and Schwartz (1954), Langstroth, McRae and Stavraky (1938) and Mangos and Braun (1966). The increase in the concentration of sodium with increasing secretory rate can be explained in the following way. When the secretory rate rises, the contact time between the precursor secretion and the reabsorptive part of the duct system decreases, therefore the fractional sodium reabsorption falls (the absolute amount of reabsorbed sodium rises) and consequently the sodium concentration in the final saliva rises.

The increasing sodium concentration with decreasing secretory rate seen in the low flow rate range could be explained by the model of Young and Schögel (1966). They suggested that in an area of the duct system situated orally to the sodium reabsorbing part of the ducts some equilibration between the interstitial fluid and the saliva could take place. This reequilibration would only be important at very low secretory rates where the contact time would be sufficiently long.

There is some evidence that the sodium reabsorptive mechanism is under nervous control. Lundberg (1955) recorded transductal potentials (lumen negative) in the cat submandibular gland but only during stimulation. If these transductal potentials

could be taken as signs of an active sodium transport, the sodium reabsorption would be under nervous control in the submandibular gland of the cat. Thus it might be possible that at very low stimulation frequencies the sodium reabsorption would be small in relation to the amount of primary secretion. This would provide an alternative or additional explanation of the increasing sodium concentration with decreasing secretory rate seen in the lower flow rate range.

Potassium

The relationship between the potassium concentration in the saliva and the secretory rate has been described by many authors. It has generally been found (Thaysen, Thorn and Schwartz 1954 and Langstroth *et al* 1938) that the potassium concentration was independent of the secretory rate, except at low rates where the concentration increased slightly with decreasing secretory rate. A somewhat similar pattern was found in the present work on cat submandibular saliva, but as seen in Fig 6 the potassium concentration increased very markedly at the lowest secretory rates. The reason why these high potassium concentrations have not been reported previously is presumeably that sufficiently low flow rates have not been studied. Recently Young and Schögel (1966) reported very high potassium concentrations (150 meq/l) in the saliva of the rat submandibular gland at extremely low secretory rates. However this gland produces saliva which never contains less than about 35 meq/l of potassium even at the highest secretory rates. In the present work it was found that in the submandibular saliva of the cat at secretory rates higher than 100 µl/min/g gland the potassium concentration was independent of the secretory rate having a value of about 10 meq/l. This value was also found by Langstroth *et al* (1938) in the saliva from the same gland.

Assuming that the primary secretion in the submandibular gland of the cat has the same composition as in the rat submandibular gland the primary secretion would have potassium concentration of about 6 meq/l (Young and Schögel 1966)

It was a theoretical possibility that the high potassium concentrations found at the low secretory rates could be due to osmotic reabsorption of water in the ducts.

Using Burgen (1956 b) figure for osmotic water reabsorption in the parotid gland of the dog it is however found, that the water reabsorption could only account for a very small part of the increase in the potassium concentration with decreasing flow rate seen at the lowest secretory rates. It is therefore obvious that an additional potassium secretion must take place in the duct system.

Transient concentrations

Burgen (1963 and 1964) described transient high sodium concentrations in the first samples of saliva taken after a resting period, from the parotid gland of the dog In these experiments only brief interruptions lasting 10—60 sec in a continuous stimulation period were used. Such brief interruptions resulted in transient changes in sodium concentration which often showed a biphasic trend with a very short drop in concentration occurring first, followed by a temporary increase The ampli

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Permeability of Brain Capillaries to Hexoses and Pentoses in the Rabbit

By

W F Agnew and Christian Crone

Recei ed 22 December 1966

Abstract

Agnew W F and C. Crone. *Permeability of brain capillaries to hexoses and pentoses in the rabbit.* Acta physiol. scand. 1967 *70* 168—175

The initial loss f the rabbit brain of non-electrolytes injected into the carotid artery was measured with Indicator Diffusion technique 3-0-methyl-D-glucose shared with D-glucose the bil y to pass int the brain by mediated transport mechanism. The rat of transport of he following bstances could not be distinguished D-mannose D-galac ose D-arabinose and D-ribose. They all penetrated more slowly tha D-glucose

D-mannitol was also lost f om the blood during the passage hrough the brain. Thi obser ion, together with the known inability of D-mannitol pass the blood-brain barrier suggest that the loss takes place nt the endothelial cytoplasma. Thus the basal plasma membrane of the endothelial cells together with the basal membrane proper may define blood-brain barrier hy terhuses

Glucose is transferred acros the wall of brain capillaries more rapidly than other non-electrolytes of similar physico-chemical structure (Crone 1965)

In view of the apparent parellelism between the permeability of the blood brain barrier and cell membranes in general t would be of interest to determine whether sugars other than glucose have the facilitating transport mechan m as in a they are known to do n the red ell membrane and in the muscle ell m m brane

Using the Indicator Diffusion technique (Chinard, Vosburgh and I in 1955, Crone 1963) the capillary permeability of the following substances w re det rmined 3-0-methyl D-glucose D-mannose D-galactose D-arabinose D-ribose and D-mannitol. To compare the pa sage of the test substances w th that of glu ose under similar circumstan es glucose wa included in the njection sol tion in all experiments. Mannitol was selected because it most unlikely that th ubstan e is tr nsferred by any mechanism other than pa sive diffusion

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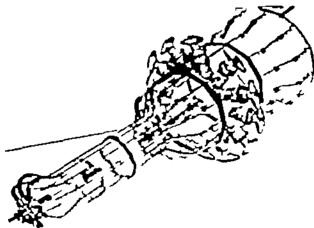


Fig. 1 Apparatus for rapid collection of blood from the superior sagittal sinus.

Methods

The Indicator Diffusion method of studying transcapillary exchange of substances during single passage through brain capillaries has been described previously (Crone 1963, 1964). It consists of rapid injection of mixture of diffusible and non-diffusible substances into the carotid artery and fast collection of blood from the sagittal sinus. Subsequently the relative loss of the diffusible substances in each sample is determined.

Animal preparation. Rabbits of either sex weighing from 4—6 kg were anesthetized with pentobarbital sodium, 35 mg/kg, administered i. as 1 solution. An anterior midline incision was made in the upper neck and tracheal cannula inserted through which the animal was ventilated with air from positive pressure respira or A branch of the right external jugular ein was cannulated for the administration of supplementary anesthesia. Mean arterial blood pressure was monitored from the femoral or carotid artery by means of mercury manometer. The left common carotid bifurcation was exposed by blunt dissection and ligature placed, but not tied, at the base of the external carotid artery. The animal was rotated unto the supine position and midline incision made in the skin of the back of the head over the posterior protuberance. A dental drill was used to make 3 mm hole over the torcula, and the animal as replaced on its back; at this point 2000 units of heparin were given and stainless steel T-cannula was inserted into the left common carotid caudal to the external carotid branch. The external carotid was then ligated with the nimal on its side and the dura over the torcula was opened with knife or dental drill. A threaded stainless steel cannula, fitting the burr hole, was screwed int the skull, and the cannula ttached to collection device. Collection of cerebral enous blood samples was started simultaneously with the injection of the isotope mixture into the T-cannula in the carotid artery. The injection lasted about 1 sec.

To collect, fast rate small samples from the rabbit torcula, the pparatus show in Fig. 1 was constructed. The collection tubes contained bout 0.4 ml each. The pparatus contained 12 collection tubes. Total collection times varied between 25 and 50 seconds. The sampling time was determined ith stop-watch.

Injection solution. Inulin was used as reference substanc instead of E ans Blue D, because it can be analysed with greater reliability especiall in he rabbi which hemolysis occurs ery readily. The injection solution (0.3—0.4 ml) contained 15 mg of inulin. This amount of inuli gav concentrations in the effluent blood between 10—100 mg/100 ml which is convenient range of concentrations for the subsequent chemical analysis. T his was added 5 μC of one of he test compounds labelled w h C-14. 15 μC of the other labelled with tritium and bout 10 mg of each of the test substances was added as arrier. The injection solution was prepared imme diatel before the experiment, nd, in most cases, was injected at room temperature. However in he later part of the series he injection solution as heated to 37 C because there w less interference ith the blood flow through the brain when this precaution was taken.

Immediately before and at he end of the experiment blood was dra simultaneously from the artery and the sinus for blood glucose determinations.

Analytical technique. Aliquots of blood samples ere precipitated with N OH-ZnSO for inulin analysis by Bojesen proced re 195. The olumes originally proposed by him ere reduced

to one-tenth, and the hydrolysis and color development took place in ml glass mpoules, the tips of which were sealed after introduction of reagent nd sample. The recovery of inulin added to blood was 97 per cent with coefficient of variation of 1.4 per cent.

Determination of radioacti ity was carried out on samples of blood precipitated with 5 trichloracetic acid. 0.2 ml of the supernatant was added t 14 ml of Bray solution (Bray 1960) containing 60 g naphtalene 100 ml methanol, 20 ml ethylene glycol, 4 g 2,5-diphenyloxazole (PPO) and 200 mg dimethyl-POPOP 1,4-bis-[2-(4-Methyl-5-Phenyloxazolyl)]-Benzene per litre dioxane. Dual counting of tritium and C-14 ctivity was accomplished in Packard *Tri-Carb* Liquid Scintillator Spectrometer (Model 3314) by counting the samples simultaneously in two channels with appropria gain and window settings. A minimum of 10,000 counts were registered on th majority of the samples.

Blood glucose was analysed according t the method described by Bergmeyer and Bernt (1963 using glucose oxidase T increase the ccuracy of the determination the concentrations ere calculated from the mean of three separat precipitations with double determination on each supernatant. This was considered necessary because the arterio-venous glucose differences were small

Th *mean transit time* (T) was calculated from semilogarithmic plot of inul concentration against time after injection as described by Lilienfield and Kovach (1956)

The *traction* (E) of the injected non-electrol tes was calculated as $(c_{ref} - c_{test})/c_{ref}$ where c_{test} is the concentration of the diffusible substances and c_{ref} is he concentration of the reference substance (inulin) The concentrations re expressed relative t the concentration in the i jection solution. The first 5—6 samples obtained were used for determination of the extraction, later samples might contain recirculating blood nd could, therefore not be used The concentrations were those in whole blood.

Several *in vitro* experiment were carried out before starting the nimal experiments and also from time time during the experimental series to determine the recovery of radioactiv material after the passage through the external collection system. This was done to mak sure that the losses of radioactiv material which occurred in the animal experiments ook place in the erebral essels of the animal and was not due t adherence rubber or glass tubes or other unknow ffects of the external system on the injection solution. These *in vitro* experiments also ensured ha he technique for determining carbon-14 and tritium in the same samples worked satisfactorily

A total of 70 successful nimal experiments were carried out.

Radioactive compounds D-arabinose D-galactose D-ribose D-mannose D-mannitol and D-glucose The Radiochemical Centre Amersham, England) C 14 labelled 3-0-meth l-D-gluc se labelled in the methyl group was delivered from New England Nucl. Corp Boston, Mass

Results

Extraction of glucose The blood glucose concentration in anaesthetized rabbit is always higher than in the wake animal The glucose concentration averaged 181 mg 100 ml (S.D = 46 n = 19)

The initial extraction of labelled glucose in 19 expts a eraged 0.27 S.D = 0.11 as com pared w th a net extraction of 0.09 (S.D = 0.05 obtained from the arterio-venous differences of glucose immediately after the end of the same experiments. The mean arterio-venous difference of glucose determined before the inje tion in the same 19 animals was 16 mg 100 ml, a value comparable to an a-v difference of glucose of 13 mg 100 ml found by Rodnight McIlwain and Tresize (1959 in conscious rabbits. The figures for the average initial isotope extraction, compared w th the net extraction values, corroborates the idea that there must be a bidirectional passage of glucose across the cerebral capillaries (Crone 1965)

The cerebral vessels in the rabbit are ery sensitive to the injection trauma and consequently large uncontrollable ariations in blood flow occurred from experiment to experiment This disadvantage, however ga e an opportunity to study the relation between glucose extraction and cerebral blood flow Fig 2 show the relation between the extraction of C-14 glucose and the mean transit time Each

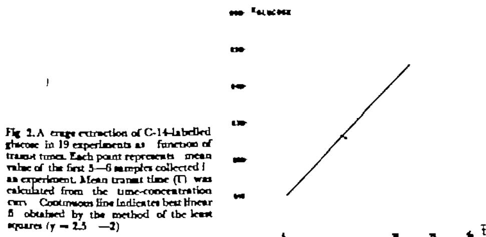


Fig 2. A erage extraction of C-14-labelled glucose in 19 experiments as function of transit times. Each point represents mean value of the first 5—6 samples collected I an experiment. Mean transit time (T) was calculated from the time-concentration curv Continuous line indicates best linear fi obtained by the method of the least squares (y = 2.5 —2)

point on the curve represent the a erage of samples from one experiment. The experiments presented on the diagram are those in which it was possible to construct a time-concentration curve and assess the recirculation with reasonable certainty By means of this relationship it is possible to convert mean glucose extractions into mean transit times.

2 *Extraction of 3-0-methyl D-glucose D-galactose and D-mannose*

LeFevre and Peters (1966) found in rat experiments that D-galactose nd D-mannose distribute in the total water compartment of the brain and though their studies did not specifically deal with the rat of exchange it is obvious from their experiments that the uptake f these substances in the brain — and therefore the passage across the blood brain barrier — takes place quite rapidly in the rat. The existence of pecial transport mechanisms was indicated by the demonstration of saturation and competition phenomena.

TABLE I. Extraction in the brain of galactose mannose and 3-0-CH$_3$-glucose compared with tha of glucose Each of the substances was injected together ith glucose

Glucose Extraction ( ) Range	Average Glucose Extraction ( ) in experiments w h			Galactose Extraction	Mannose Extraction	3-O-CH$_3$-Glucose Extraction
	Galactose	Mannose	3-O-CH Glucose			
10—20	15.5	15	14.9	11.6 = 16	12.7 (= 18	17.6 (= 4)
20—30	25.2	23.8	25.6	21.6 (= 7	20.4 (= 7)	4.9 (= 7)
30—40	33.7	—	34.0	33.5 = 9	—	32.9 (n = 9

TABLE II Extractions in the brai of arabinose nd ribose compared with that of glucose. Each of the pentoses was injected together with glucose

Glucose extraction ( ) Range	Average Glucose extraction ( ) in experiments with Arabinose	Ribose	Arabinose extraction	Ribose extraction
0—10	6.0	5 C	3.3 (= 12)	2.3 (= 11)
10—20	—	15.1	—	11.6 (= 14)
20—30	24.8	24 6	20.8 (= 5)	21.. (= 5

TABLE III Extractions in the brain of mannitol and glucose in experiments where mannitol and glucose were injected together

Glucose extraction ( ) Range	Average Glucose extraction ( ) in Mannitol experiments	M nitol extraction
0—10	5.7	3 7 = 3)
10—20	15 4	10 8 = 19
20—30	23.7	18 2 = 12)
30—40	35.2	28 7 = 25

In the present series of experiments the rate for 3-0-methyl-D-glucose losely followed that of glucose whilst galactose and mannose left the blood more slowly (Table I)

The figures are grouped in three classes according to the glucose extraction Each class covers a certain range of mean transit times of the injection solution f 1 g ?) The glucose extraction in each of the three groups was the same regardles of which other test substance was present. This indicates that the glucose passage was independent of the presence of the other hexoses.

3. *Extraction f D-arabinose and D ribose* The results of experiments with these two pentoses are shown in Table II The a erage glucose extraction n the tw groups lie close to one another indicating that the presence of the pentose in the jection solution does not affect the transport of glucose The extractions of the pent ses were clearly below those of glucose at high flow rates.

4 *Extraction of D-mannitol* The extraction of mannitol wa lower than that of glucose (Table III) the difference being more pronounced at hig flow rates where the mannitol extraction was 67 of the glucose extraction.

TABLE IV. Average ratios between the extractions of various nonelectrolytes and those of glucose. The figures are arranged according to the glucose extractions which reflect variations in mean transit time of the injected solution.
Ratio $E_{test}/E_{glucose}$

Glucose extraction range (%)	D-Galactose / D-Glucose	D-Mannose / D-Glucose	CH_3-O-Glucose / D-Glucose	D-Arabinose / D-Glucose	D-Ribose / D-Glucose	D-Mannitol / D-Glucose
0—10	—	—	—	0.55 (S.D. 0.39 n = 12)	0.38 (S.D. 0.23 n = 11)	—
10—20	0.68 (S.D. 0.21 n = 16)	0.74 (S.D. 0.15 n = 18)	1.18 (S.D. 0.06 n = 4)	—	0.76 (S.D. 0.24 n = 14)	0.67 (S.D. 0.36 n = 19)
20—30	0.84 (S.D. 0.20 n = 7)	0.72 (S.D. 0.19 n = 7)	0.97 (S.D. 0.04 n = 9)	0.92 (S.D. 0.25 n = 5)	0.85 (S.D. 0.35 n = 5)	0.75 (S.D. 0.[illegible]7 n = 12)
30—40	1.00 (S.D. 0.06 n = 9)	—	0.95 (S.D. 0.04 n = 9)	1.02 (S.D. 0.05 n = 4)	—	0.81 (S.D. 0.25 n = 23)

TABLE V. Clearance index (extraction × the reciprocal of the mean transit time) at three different average glucose extractions (compare Table III)

T sec	Average glucose extraction	Clearance index: Glucose	Clearance index: Mannitol
8.1	15.4	1.89	1.34
11.9	23.7	1.99	1.33
17.2	35.2	2.02	1.66

Clearance index = E · (1/T), where E is extraction and T is mean transit time.

Table IV gives the extractions of various substances relative to the extraction of glucose. This mode of presenting the results emphasizes the observation that the differences in rate of passage between glucose and the other substances disappears when there is marked reduction in flow.

Only 3-O-methyl D-glucose can safely be said to share with glucose the ability to pass the cerebral capillaries by a facilitating mechanism.

3. *Clearance of glucose and mannitol.* Table V shows the calculated clearance indexes of glucose and mannitol at various rates of blood flow. The clearance index is calculated E · 1/T. This product is proportional to the clearance E × Q (where E is extraction and Q is blood flow) because Q is proportional to 1/T. The average values of glucose extraction in the mannitol series (Table III) were used to calculate the mean transit time of the test substance (see Fig. 2). The results show a slightly increasing clearance index with falling blood flow.

Discussion

The experiments were carried out in order to test LeFevre and Marshall s hypothesis (1958) that the ability of a nonelectrolyte to pass cell membranes by mechanisms other than diffusion depends on the conformational shape of the molecule The differences in transport rate of the various sugars tested in the present study were however so small that it would be unjustified to draw any conclusions with regard to this aspect of the uptake from blood into brain tissue. Only one substance, 3-0-methyl glucose, can safely be said to share with glucose the ability to pass into the brain by a mediated transport mechanism. It is possible that the quite large amounts of carrier substance used have, in fact, led to saturation of a transport system so that differences in passage rate have become less prominent Experiments carried out at lower "loads of the uptake system might have disclosed differences in the behaviour of the different pentoses and hexoses used. The fact that glucose was always present may also have interfered with the uptake of other sugars.

The linear relationship between glucose extraction and mean transit time (Fig. 2) makes it clear that the uptake of glucose in the brain is diffusion-limited At very low blood flow it may however become flow limited In this respect glucose behaves as all other polar substances taken up by any tissue

The observation that the ratio between the extractions of the test substances and glucose (Table IV) approach one as the blood flow falls is explained by the fact that the extraction of diffusible substances always approach the same value at low rates of blood flow where the loss of even highly polar substances become blood flow limited (Fig 3) Renkin (1955)

The clearance of the test substances tended to increase with falling blood flow (Table V) This was a somewhat puzzling and unexpected finding The most probable explanation is that new capillaries open up due to autoregulatory mechanisms in the brain. An increased surface area will tend to increase the clearance despite a fall in blood flow This phenomenon has also been observed in muscle tissue (Renkin 1959 1966)

It is still not unequivocally settled whether the diffusion hindrance between blood and brain tissue resides in the endothelial cells or in the apposed glial investments. Electronmicroscopic studies (Reese 1966) show that the endothelial cytoplasm in the brain capillaries can engulf protein molecules such as ferritin, but that the material never proceeds across the basal membrane The present results lend support to the hypothesis that the luminal membrane of the endothelial cell exhibits some selectivity against small molecules of almost similar size and physico-chemical structure but

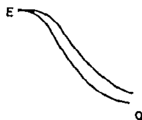


Fig. 3. Curves showing the general relation between blood flow and extraction of two substances which differ in ability to pass through capillary wall

that the luminal membrane does not behave as a barrier in the true sense of the word. The well known fact that mannitol does not penetrate into the brain from the blood (Park, Johnson, Wright and Batsel 1957) together with the findings reported above that mannitol is lost from the blood strongly suggest a difference in permeability between the luminal cell membrane and the basal cell membrane of the endothelial cells in the brain capillaries. Thus, polar substances are permitted to pass into but not beyond the endothelial cytoplasm.

Another explanation of the findings may be that mannitol is lost to an extra vascular space which is so small that it cannot be distinguished from the vascular volume. Experimental evidence of the existence of such a space has been put forward by Agnew (1965)

It is not possible at the present time to carry the discussion about this point further. The movement of substances from blood to brain tissue must involve transcellular transport, because the composition of the brain interstitial fluid is regulated. The basal plasma membrane of the endothelial cells together with the basal membrane proper might be the important structures defining blood brain barrier characteristics. The possibility that the extravascular glial investment is responsible cannot be excluded, though the fact that this structure is not continuous makes this less probable

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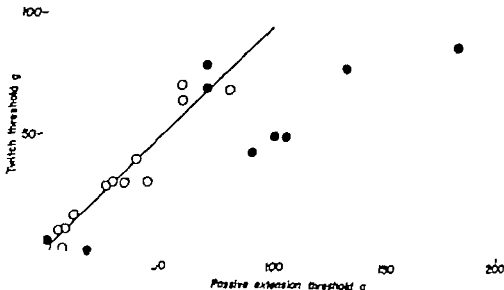


Fig 2. Scatter diagram of observations of thresholds to passiv extension (bscissa) nd isometric twitch contraction (ordinat) in 22 anterior tibial tendon organs. Each point represent the observation of one unit. *Filled circles Tendon organs in the proximal part of the muscle. Empty circles* Tendon organs in the distal part of the muscle

tension rise can be registered, and could equally well be recorded when the muscl is contracting isometrically as when it shortens with zero load.

B Tension thresholds

The threshold to active twitch contraction was determined for 22 anterior tibial tendon organs Fig 1 b) In order to avoid interference from early discharge spikes *the threshold was chosen as the minimum twitch contraction setting up a discharge* of greater than 2 5 msec latency from the start of contraction. This would en ure that the spike originated in the muscle at some time after the onset of tension rise Active twitch thresholds aried from 0—90 g mean 38 g Zero threshold refer to four tendon organs firing ponta eously at no recognizable muscle load

In contrast to the beha iour of soleus tendon organs Jansen and Rudjord 1964) all anterior tibial units responded to passive tretching of the muscl w hin its physiological limit For instance the tendon organ shown in Fig 1 seen t discharge at 40 imp sec at full ph siologic extension the muscle tension being 130 g (Fig 1 d) Correspond ng maximal pa si e tension alues varied f om 1 ≠ 300 g in different experiments The tension at the first sensory mpul from the Golgi *tendon organ was measured during linear t etches at 18 mm sec* up to full physiologic extension in the manner described by Jansen and Rudjord 1964) Such passive extension thresholds aried between 0– 180 g mea 10 g

It appears from Fig 2 that most tendon organs in the present seri had nearly the same thresholds to passive stret h and activ tw tch contraction ndicating a similar tension distribution at the tendon organ tes under the two ircumstances

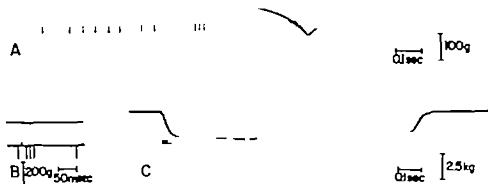


Fig 3. A and B Activity of spontaneously discharging Golgi tendon organ during passive linear stretch up to full physiologic extension (A) and during minimal muscle twitches (B) C Golgi tendon organ response to fused tetanic contraction of muscle.

Six units had appreciably (> 20 g) lower twitch tension than passive extension thresholds. When localized in the muscle by mechanical pressure these tendon organs were all found to be situated in the proximal part of the muscle (Fig 2) This finding corresponds to the distribution found in the soleus muscle for tendon organs not discharging at full passive extension, and supports the explanation of Jansen and Rud jord (1964) of differences in the exciting force in differently located tendon organs. It would seem that tendon organs situated in the proximal part of the muscle would not necessarily carry the same local load during passive muscle stretching and active muscle contraction However a tendon organ directly in series w th the common muscle tendon, would register approximately the same tension in the two instances. The four spontaneously discharging tendon organs were localized one in the proximal and three in the distal part of the muscle. The responses of ne of the latter are illustrated in Fig 3.

It appears from Fig 3 A and B that th firing frequency of this tendon organ increased with ery slight increases in tension However the dynamic and static sensitivities of pontaneously discharging tendon organs as later described, were found to be within th normal range of ariation for the anterior tibial series. "Spontaneously" firing units were not found in the soleus muscle J nsen and Rudjord 1964)

C. Static tendo organ properties

Th static sensitivity to tension sensitivity to absolute tension level) was deter mined for all nterior tibial tendon organs during a fused tetani contractions graded entral root stimulation at 150 H , Fig 3 C and b maintained level of passi stretch, n the manner described by Jansen and Rudjord 1964) As appears from Fig 3 C there was some difficulty in mai taining a constant tetanic tension level, due to the fat guability of this fast contracting muscle Accordingly the steady state frequency was measured at 0.3 sec following the onset of contraction. The

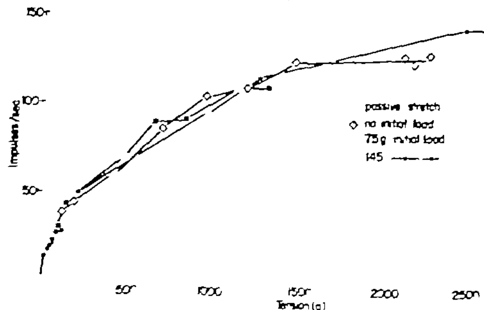


Fig. 4. Frequency tension diagram of the Golgi tendon organ shown in Fig. 3 C. Steady state measurements made at different levels of passive extension and during fused tetanic isometric contractions at 3 different levels of initial extension.

tendon organs studied showed the same low rate of adaptation as that found for soleus tendon organs by Jansen and Rudjord (1964).

In the unit illustrated in Fig. 3 C the frequency decayed by 10.6 % during the period 0.3—1.3 sec following a ramp stretch to full physiologic extension. The mean value for units firing at more than 20 Hz, was 15.2 % (7.4 — 22.0 %).

Fig. 4 shows the steady state frequency tension plot of the tendon organ also illustrated in Fig. 3 C. Similar plots have been made for all anterior tibial tension organs investigated. The typical curve of the series was found to be one where an initial steeper part merged continuously into a less steep part at higher tension values. *Curves for tetanic contractions at various levels of initial stretch (load) overlapped,* consistent with the fact that the tendon organ registered a constant fraction of total muscle tension. However, 4 curves showed the same kind of displacement to the right with increasing pretetanic load as has been found to be characteristic of most soleus tendon organs (Jansen and Rudjord 1964). These units were situated in the proximal part of the anterior tibial muscle and were among the group with lower twitch thresholds described earlier. During fused tetanic contraction superimposed on maintained levels of passive stretch, these tendon organs registered mainly the active tension component. This again can be explained by the hypothesis of *different tension distribution* at local muscle tendon junction discussed above. Although it was often possible to fit straight lines to parts of the frequency tension plots, e.g. from 500—1,500 g, the complete curves were clearly never rectilinear. This makes comparison of sensitivity values with those given by Jansen and Rudjord (1964) for soleus tendon organs difficult. However, approximate slopes for the tetanic part of the frequency

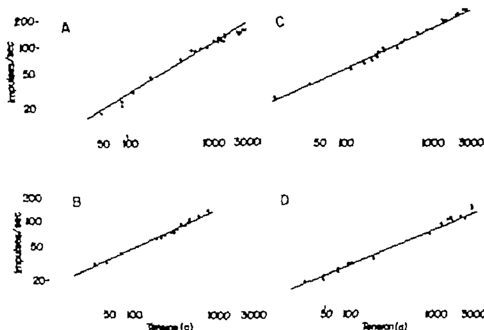


Fig. 5. A. Frequency/tension diagram shown in Fig. 4 plotted on log-log scale. B—D. Responses of three additional anterior tibial units. Straight lines have been fitted by eye

tension plots have been found to vary from ca. 4—ca. 15 Hz/100 g in different units. This agrees well with the sensitivity range found for soleus tendon organs, and indicates that the static sensitivity to tension in the 500—1,500 g area is approximately equal for tendon organs in the two muscles

The shape of the frequency/tension curves such as of Fig. 4 suggests that they may be described as power functions. This is supported by the approximate straight line relationships obtained when the same data are plotted on log-log coordinates as in Fig. 5. Fig. 5 A corresponds to the unit illustrated in Figs. 3 C and 4

Accordingly the steady state response of the anterior tibial tendon organs is approximately described by an equation of the form

$$R = K \cdot P^n \tag{1}$$

where R is the response frequency, K a constant, P the muscle tension, and n a constant determining the slope on a log-log plot. Clearly the exponent n is a better characteristic of the complete frequency/tension relationship of the tendon organs than linear slopes of subdivisions of the frequency/tension curve such as given for soleus units by Jansen and Rudjord (1964). In 19 units where a complete frequency/tension curve could be drawn n was found to vary from 0.41—0.63 with a mean of 0.49

The different general form of frequency/tension plots of the anterior tibial and soleus tendon organs as given in the present study and by Jansen and Rudjord

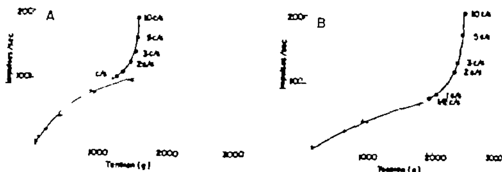


Fig 7 Frequency/tension diagrams of tendon organ responses. ( ) Steady stat responses measured as (Fig 4 (^) Peak responses during sinusoidal stretching A Anterior t bial unit show in Fig 6 A—C. B Soleus unit show in Fig 6 D—F

Fig 6 is given in Fig 7 It appears that peak frequency responses during slow (1 Hz) cycles correspond to steady state values for the same tension whereas the peak frequency at higher rates of stretch overshoots that expected from the tension developed.

When comparing the relationship between peak frequency of firing and rate of sinusoidal stretch of all units, no characteristic difference was f und between anterior tibial and soleus tendon organs, and their dynamic responses appeared to fall within the same range of ariation The similarity of the dynamic sensitivities of tendon organs in the two muscles is further supported by the observation that the dynamic overshoot during anterior tibial tetanic contraction tended to disappear in th fati gued muscle, when its rate of rise of tension approximated that of normal soleus muscle.

Discus ion

A more complete description of the response of the tendon organs would be of great interest and convenience. The transfer function approach provides possible methods for such a comprehensi e description (Machin 1964) This analysis ca only be carried out with exactitude on receptors with linear input/output relationships As demonstrated above the tendon organ response is a nonlinear function of m scle tension (Fig 4) However the upper range of such response curves (500— 1 [illegible] g) can approximately be fitted by straight lines, and for soleus tendon org n this applies to an even greater part of their range (Jansen and Rudjord 1964 Thi approximate linearity over an appreciable range just fies an attempt to determine the transfer function of the tendon organs from th tendon organ characteri tics found during dynamic stimulation in this area. If uccessf l t merit would be more accurate description of the dynamic as well as the tatic properties of the eceptor in the 500—1,500 g range

If the fused tetanic contraction of the fast contracting anterior t bi l muscl is regarded as a step tension input, and the dynamic overshoot Fig 6 A a the transient response of the receptor the time course of th transient conta the

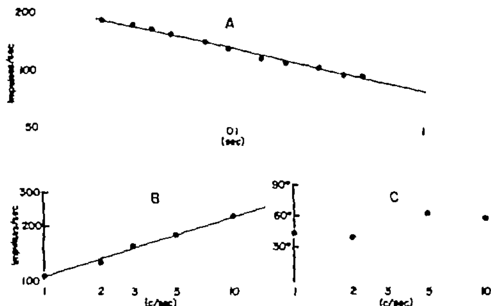


Fig 8 A Time course of dynamic overshoot recorded in Fig. 6 A plotted on log-log scale. A straight line has been fitted by eye. B Impulse frequency plotted against sinus stretch frequency on log-log scale. A straight line has been fitted by eye. C Phase lead of peak impulse frequency on peak muscle tension plotted at different rates of sinusoidal stretch.

information about the dynamic properties of the receptor. Admittedly these are rather crude approximations. As shall be seen, however, they lead to certain simple and verifiable predictions of receptor properties. In Fig 8 A the time course of the dynamic overshoot of the receptor shown in Fig 6 and Fig 7 A has been plotted on log-log coordinates. The curve is reasonably well described by a straight line which has been drawn on the plot by eye. Similar results were obtained for the transient responses of the other anterior tibial tendon organs investigated.

This is analogous with the adaptive properties of the slowly adapting stretch receptor of the crayfish (Brown and Stein 1966), the transient response of which is described by the equation

$$R = a P t^{-k} \tag{2}$$

In this equation R is the response measured as frequency, a is a constant determining the sensitivity of the receptor to unit change in tension (P), t is time, and k a constant describing the time course of the transient response. On log-log plots such as Fig 8 A, k can be measured directly as the slope of the regression line. From (2) the transfer function of the receptor can be derived (Chapman 1963, Brown and Stein 1966).

The formulation of a transfer function leads to two simple predictions (Brown and Stein 1966) which can be controlled in the present material

1 The peak firing frequency during sinusoidal stretching should increase as the k^{th} power of the stretching frequency

TABLE I

	k as determined from transient	k as determined from peak frequencies	k as determined from phase lead at 2 Hz
Anterior tibial	0.32	0.34	0.43
	0.34	0.26	0.2[illegible]
	0.27	0.32	0.33
	0.64	0.74	0.61
Soleus		0.5	0.39
		0.60	0.50
		0.28	0.2[illegible]
		0.34	0.33

2 The phase lead of the response should be constant and equal to k 90

In Fig 8 B the peak frequencies of the anterior tibial unit previously shown in Figs. 6 and 7 have been plotted against stretch frequency on a log-log scale It appears that the power function relationship predicted by Brown and Stein is supported, and a straight line *has* been fitted to the diagram by eye, the slope of which is another measure of k.

In Fig 8 C the phase of peak firing frequency on peak tension are presented for the same tendon organ. As discussed in detail by Brown and Stein (1966) these measurements are necessarily rather inaccurate particularly at higher stretch frequencies. In our material there did not seem to be larger scattering of phase lead values at different stretch frequencies, than that inherent in the measurement uncertitude Probably the measurement at 2 Hz is as representative as is possible under the given experimental conditions.

The three independent estimates of k are presented in Table I for four anterior tibial tendon organs submitted to frequency analysis k-values are also given for *four soleus tendon organs. Since no approximate transient response occurred for* tendon organs in this muscle k was determined from the peak frequencies during sinusoidal stretching and phase leads, only

There appears to be a fair amount of agreement between the values of *k* derived by the different methods The material is too small to cover the entire range of variation in tendon organ properties, but it appears that the values for anterior tibial and soleus tendon organs are rather equal

Thus the conclusion of the two preceding sections of this investigation on the similarity of static and dynamic properties of Golgi tendon organs in the soleus and the anterior tibial muscles is supported But it should be emphasized again that the transfer function of the tendon organs remains a rather crude approximation on account of the non-linearity of the receptor

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On the Firing Pattern of Spinal Neurones Activated from the Secondary Endings of Muscle Spindles

By

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Abstract

JANSEN, J K S K NICOLAYSEN and T RUDJORD. *On the firing pattern of spinal neurones activated from the secondary endi gs of muscle spindles* Acta physiol. scand 1967 0 188—193

Some properties of group of pinal neurones ith axons ascending in the dorsol teral funicle are reported. They re excited by muscle stretch, and pause during muscle con raction Their threshold to electrical stim lation of he muscle nerv is the group II ra ge Their la encies re grea er tha that of the group I activated units Their firing frequency increases pproximatel linearl h muscle length. Their discharge pa ern is haracterized by relati ely high degree of regula I is suggested tha they represen system relaying information on the length of individual muscles or synergistic muscle groups to the brain

Introduction

The existence of a group of units running in the dorsolateral funicle of the pinal cord and act ated from the secondary endings f muscle spindles was reported in a prel minary communication (JANSEN and Rudjord 1965) It is appreciably more difficult to obtain stable records from these axons than from other types of unit in the dorsolateral funicle of the cord. However in the course of a investigation of the group Ia activated un t w th axon scendi g in this part of the cord Jansen, Nicolaysen and Rudjord 1966 a certain mount of additional information has been obtained also on the group II activated units Since these group II unit differ characteri tically in their f ring pattern from the group Ia nits previously examined (Jansen *et al* 1966 and sin the properties of thi particular group of un t ha not yet been fully described, th material even though l mited, appear t deserve a systematic description

Methods

The experiments were performed on Nembutal anesthetized One hind leg w denerv ted except for the muscles under study which were the gastrocnemius-soleus (S nd he tibiali erior and extensor digitorum longus muscles T EDL The en ral root L VI L VII nd SI were al

cut. The muscle tendons were connected to an isometric myograph which could be moved at constant velocity (17 mm/sec) to any desired position. The activity of single axons was picked up by micropipettes in the ipsilateral dorsolateral funicle and recorded on moving film and magnetic tape. Full details about the experimental arrangement and treatment of the data have been given in a preceding paper (JANSEN *et al.* 1966).

Results

Identification of units. All the eight units of the present report were activated by passive stretch of either the GS or TA EDL muscles. None of them were activated from both groups of muscles. They were all fired by electrical stimulation of the appropriate muscle nerve close to the muscle. Their threshold to electrical stimulations was always greater than that causing a maximal contraction of the muscle, for some by as much as a factor of four. Their latencies to single shock stimulation were from 4.5 to 7 msec. An example is shown in Fig. 1 A. At threshold their latencies might be rather variable from trial to trial but on increasing the shock intensity their time of firing would become regular. The high threshold and long latency of these units serve to distinguish them from the Ia DSCT units of the dorsolateral funicle. The latter all have latencies between 3 and 4.5 msec and thresholds less than α maximal, frequently less than α threshold (Jansen *et al.* 1966). The discharge of the group II units was characteristically interrupted during the peak of a twitch contraction of the muscle (Fig. 1 B). This indicates that they were not appreciably activated from the tendon organs of the muscle.

Response to stretch. The response of a typical unit to a linear stretch of the muscle is illustrated in Fig. 1 C. Its discharge frequency increased with increasing length, and there was only a small reduction in firing frequency at the transition from dynamic stretch to static extension. This feature distinguish the present group II activated units from the Ia units previously described (Jansen *et al.* 1966). A certain measure of the dynamic sensitivity of a unit can be obtained from the difference in firing frequency at the end of the dynamic stretch and during maintained extension at the same length. This is the so called dynamic index (Jansen and Matthews 1962 a). For the eight group II activated units of the present study the mean dynamic index was only 15 imp/sec at a stretch rate of 17 mm/sec. This is a value appreciably smaller than that of the Ia activated units determined under similar conditions (JANSEN *et al.* 1966) and it supports the notion that the group II activated units receive their main excitation from the secondary endings of the muscle spindles.

Other typical features of the behaviour of the group II activated units appear from Fig. 1 C. They usually showed a resting discharge when the muscle was completely slack. This applied to all but one of the eight units examined. The resting discharge was always fairly regular and its frequency varied between 5 and 15 imp/sec. The firing frequency of the group II units at maximal extension of the muscle was usually less than that of the Ia activated units of the same muscle, but two of the present units fired at frequencies of about 80 imp/sec at full extension and this is about in the middle of the range of the group Ia units.

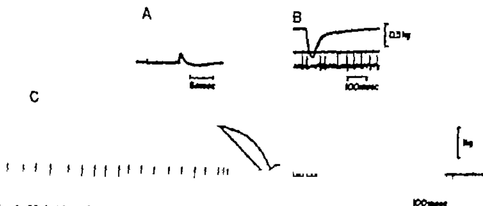


Fig 1 Unit identification nd response to stretch. A. Axon spike in dorsolateral funicle elicited by single shock stimulation of GS nerve. Internal y 1 5 times maximal. Superimposed sweeps. B. Same unit, firing pattern during GS twitch contraction Initial tension 200 g C. Same unit Response t a linear stretch of GS t a length bout 3 mm less tha full physiological extension. Length and tension traces overlapping initially During stretch length trace below tension trace Spikes retouched.

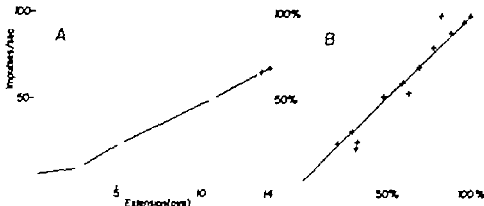


Fig 2. A. Frequency-extension relation of GS unit. B Normalized observ tions on the frequency extension relation of 5 GS units. Ordinate firing frequency as percentage of he frequency increment due to stret h Abscissa degree of extension as percentage of final alue Data from the last 10 mm of stretch for each uni

Frequency-length elation. The mean frequency of firing at various muscle lengths was determined during a series of progressive short stretches. The frequency was measured 0 5 sec after each d namic stretch so that the values should be comparable to those of the secondary endings determined previously Jansen and Matthews 1962 b Alnaes, Jansen and Rudjord 1965 An example f the results is illustrated in Fig 2 A. It appears that the firing frequency increased approximatel linea ly with increasing muscle length over the greater part of the range The esponse curve was usually less steep at the smallest lengths In this range there m ght ll be some lack in the muscle and some of the secondary end ngs migh t not et have reached their threshold An approximate linearity of this relation was obtained for all the five units studied in this way The slope values aried between 2 and 5 imp sec mm and this is within range of variation of the slope values of the secondary endi gs of the

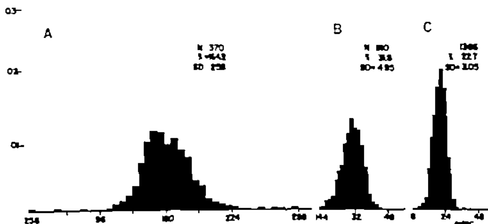


Fig. 3. Histograms of intervals between consecutive impulses t three different levels of ctivity (A, B, C) The ordinate gives the probability density of intervals of the duration given on the bscissa (msec) Interval classes 6.4 msec in A, 1.6 msec in B and C. Total N of observ tions (N), mean interval (X) and standard deviation (SD) are given for each histogram.

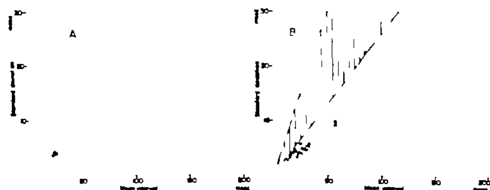


Fig 4 Relationship between standard deviation of interval distributions and mean interval. A. Data from one G8 unit B Dat from six different units, each indicated by separate symbol. Shaded area represents the degree of ariability found in Ia DSCT units (Jansen et al. 1966)

same muscles The entire material is presented in normalized way in Fig 3 B and it ppears that the deviations from linearity are moderate and probably not important t the present level of description

Distribution f firing intervals O e of the characteristic features of the firing pattern of the group II type of units was the relatively high regularity This also ppears from the distribution of firing intervals during arious levels of maintained stretch. Examples from one cell re shown in Fig 3 The distributions are all unimodal and fairly symmetrical This pplies to all the six units for which uch observations were obtained, and these six cells were examined at 38 different levels of activity Altogether these interval d tributions differ from those of the Ia type of units (Jansen et l 1966) by their smaller degree of ariability

Relationship between variability and mean interval. As pointed out by Werner and Mountcastle (1963) and Stein (1965) this relationship is a most important parameter in the description of neuronal behaviour. The data from one cell are presented in Fig 4 A. There appears to be an approximately linear increase in the standard deviation of the distributions with increasing mean intervals. This simple relationship is in general confirmed by the data from the five other units examined. Our entire material is presented in Fig. 4 B. In this graph, each unit is represented by a separate symbol. Certain deviations from the linear relationship are apparent but they do not appear to be systematic. As an initial description therefore, the linear relationship between standard deviation of distribution and mean interval appears as a fair representation of the material within the range examined.

To illustrate the difference in behaviour the range of variability of the Ia type of units described earlier (Jansen *et al.* 1966) has been shaded in Fig. 4 B. The present units all occupy the region below and it is of interest to point out that higher order sensory neurones can fire with a coefficient of variation $\left(\frac{SD}{\text{mean interval}}\right)$ as low as 10 per cent (Fig. 4 B).

Serial dependency of duration of intervals. This feature of the discharge pattern has only been examined as the duration of the mean interval in the conditional interval distributions. In this kind of analysis, which has been performed on the data from six group II type of units, negative serial correlation between the duration of neighbouring intervals appears as decreasing mean interval of the conditional distribution with increasing duration of the conditioning interval (Jansen *et al.* 19[illegible]). This was a very prominent feature of the Ia type of units but much less conspicuous in the present material. At low levels of activity there was no apparent serial correlation in the interval series. At the highest levels of activity, however, four of the cells showed a consistent and systematic increase in conditional mean interval with decreasing duration of the conditioning interval. This indicates the existence of a negative serial dependency also in the discharge of group II units, but its degree was less than that of the Ia units, and probably not of interest to explore further in the present limited material.

Discussion

The nerve cells examined in the present investigation appear to be activated from the secondary endings of the muscle spindles without appreciable additional activation from group I afferents. The evidence for this statement was given in the result section. The secondary endings are less dynamically sensitive than the primary endings and their afferent signal can usefully be described as a measure of muscle length (Matthews 1964). In their linearity (Fig. 2) and small dynamic sensitivity (Fig. 1) the units of the present study resemble the secondary endings and they can accordingly be said to mediate information on muscle length to some region of the brain. It should be pointed out that although the axons of the present type II units run in the ipsilateral dorsolateral funiculus of the spinal cord among the axons of the dorsal spino-cerebellar tract there is at present no direct evidence concerning their area of termination.

Spinal neurones with ascending axons and activated from group II muscle afferents have been reported previously (Oscarsson 1958, Lundberg and Oscarsson 1962).

The present units differ from the units of the "ventral spinobulbar tract" of Lundberg and Oscarsson (1962) in that they run in the dorsal part of the lateral funicle. The ventral spinobulbar neurones, furthermore, are activated from group III and skin afferents in addition to group II afferents and there is a large amount of convergence from different muscles to each neurone. The present units were all activated from either the GS or the TA EDL muscle, never from both. One of the units was even activated from EDL exclusively and unaffected by stretch of the TA muscle.

A final point of more general interest concerns the relatively high degree of regularity of firing of the present type of units. An elaboration of the quantal excitation model of Stein (1965) was found to account for the firing pattern of the Ia DSCT neurones (Jansen *et al.* 1966). Referring to the same type of model the regularity of the type II units suggest that their unitary input EPSPs are appreciably smaller than those of the Ia units.

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Effect of Prolonged Heavy Exercise on Renal Function and Urinary Protein Excretion

By

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Abstract

CASTENFORS, J., F MOSSFELDT and M PISCATOR *Effect f prolonged heav ercise on renal function and urinary protein excretion* Acta physiol scand 1967 0 194 – 206.

The urinary proteins in normal men participating in an 85-kilometre ski race held 1964 and 19 5 were examined by electrophoresis, immunoelectrophoresis nd gel filtration The urinary excret n of protein, glucose ribonuclease and α-amino cid-N was also studied. I conjunction th he 1965 race additional studies were made of urine flow free w ter clearance crea inine learanc sodium, phosphorus and potassium excretion nd examination of urinary sed ment There as significant increase in total protein and albumin excretion during he race The protein excretion aried considerably from one individual to another I 9 subjects who partic pated in bo h years the protein excretion, and lso the percentage albumin found by electrophoresis, showed g nificant correlation between the tw years The excretion of glucose nd α-amino acids, hich ar freely filtrable in the glomerule as not significantly changed Nor was he excretion of the low olecular weight protein, ribonuclease hich ma be expected to be almost omplet l filtrabl suggests tha tubular reabsorption is mainly unchanged d n g exercise but tha glomerular permeabilit for rela ively large molecules is increased Creatinine le ranc decreased during the rac Urine flow and sodium excretion decreased slightl during exercise Potassium ex n increased both during and after the ski race Urinary sedimen showed inc eased amounts d ed and whit cells and h line casts

The ncreased urinary protein excretion after muscular exercise has been kn wn for a long time *Leube 1878 The distribut on and nature of the urinary pr teins* after exercise has been tudied by several in estigators Nedbal and Sel ger 1958 Poortmans and an Kerchove 1962 These tudies showed that the n re se n protein was principally albumin The mecha isms responsible for th incr ase are poorly understood. Man theories ha been proposed mechanical trauma Barach 1910) acidosis (Bjure 1925 changes n the renal cir ulation Starr 1926 l ylor 1960) Most studies on protein excretion during exercise ha been don during heavy exercise of short duration and related to protein excretion onl W nsidered it of interest to in est gate the effect of prolonged exhau ting m scula w rk on protein excretion and also on the glomerular and tubular function

TABLE I Mean values and range for age weight, height, physical working capacity loss of weight and skiing time in the 1964 and 1965 test groups.

Material	Age years	Height cm	Weight kg	Physical working capacity[1] kpm/min	Loss of weight kg	Skiing time[2] min
1964	37	179	75	1.515	1.90	563
group = 20	23—62	172—197	62—84	1.300—1.800	0—3.5	458—694
1965	39	179	74	1 493	1 78	436
group = 15	24—63	165—190	60—86	1.300—1.800	1.0—3.1	341—573

Defined as physical working capacity at heart rate of 170/min under standardized conditions on bicycle ergometer (Sjöstrand 1947)

Calculated only for the 9 subjects who participated both in 1964 and 1965.

Material and methods

The studies were made in 1964 and 1965 in conjunction with the Vasaloppet which is an annual ski race in Sweden. The race covers distance of 85 km in hilly errain, the distance being the same every year The surface for skiing was better in 1965 tha in 1964 The skiing time was bout 7—12 hrs in 1964 nd about 6—10 hrs in 1965 T enty subjects were studied in 1964 and 15 in 1965. Nine of the subjects participated on both occasions Age, physical orking capacity (W_{170}) skiing time and loss of weigh in he 1964 and 1965 groups are show in Table I All participants in the study were well trained skiers, mostly doctors, in good physical health.

I the 1964 study only the urine tha was oided at the end of he race was collected I the 1965 race urine as collected on three occasions, before, on completion and, from some subjects, some time fter completion of the race All of the urine collected was spontaneously oided Both in 1964 and 1965 the subjects were allowed free access fluid hroughout the study The first sampling (period I included the overnigh urine of 11 subjects preceding the race Four subjects had discarded heir overnight specimens, so tha the period in these cases was of short duration The second sampling period II included all the urine produced during the race by 10 subjects and, from 5 subjects, the urine produced only during the last part of he race 1 5—5 hrs The urine from period II was collected bout 20 min after arrival at the w nning-post I 10 subjects it was also possible to collect urine produced during third period (period III of arying duration (5—16 hrs after exercise This period included dinner nd high fluid intake

The urine as collected in plastic bottles with sodium azide as preservati and was frozen until nalysed I he 19 study samples of fresh urine ere centrifuged and the sediment was immediately examined macroscopically

Venous blood samples ere taken in the morning before the race and again bout 25 min after arriv l at the winning-post The enous blood samples were collected in heparinized tubes, centrifuged immediatel room emperature and the plasma was frozen until analyses were performed

I the 1964 study no med urine collections were made The excretion of urine components as expressed per 1 000 mosm, which was the only analysed reference tha could be expected to be reasonabl stable I he 1965 stud the excretion of protein was calculated per minute, per g of creatinine and per 1000 mosm The correlation coefficient for protein min and protein gram creatinine as = 0 78 P 0 01 and for protein mm and protein 1 000 mosm = 0 72 (P<0.01) The excretion in the 19 5 study is expressed per gram of creatinine because of the large dead space error during these rather short collecting periods and the lack of adequately timed collections in

some subjects. The data from the subjects with unreliable time information (tw in period I tw in period II and four in period III) were excluded from the calculation of urine flow osmolar lear ance, free water clearance and creatinine clearance

The urine from one subject was collected only once at the end of the race without indication of time, and therefor all of his alues re excluded from the statistical analyses of the 1965 group.

Osmolality in urine nd plasma was determined by a modification of method described by Bowman, Trantham and Caulfield (1954) The plasma osmolality measured before the rac was used for calculation of osmolality learance and free water clearance in period I nd the pla ma osmolality at the end of the race was used for this calculation in period II There was not enough plasma for osmolality determination i the case of 4 subjects in period I nd of 6 ubjects in period II Osmolal nd free w ter clearance in these subjects were estimated on the basis of the mean alue for plasma osmolality of the other subjects. I the third period, in which no plasma sample as obtained, the ndi idual osmolality value used in period II was used for the calculation.

Creatinine i plasma and urine was determined by the method described by Hawk, Oser and Summerson (1947) as modified for the uto-analyser. Plasma creatinine determinations for period I were not done in four subjects and for period II in three subjects. The mea serum creatinine level in the other subjects for these tw periods was used for calculating the creatinine learance and urine/plasma creatinine ratio in these subjects. I period III in which no plasma sample was obtained the individual plasma level used period II was used for the calcula ion.

Sodium and potassium were determined in plasma nd urine with a flame photometer. Insufficient plasma for determination of the sodium concentrations in periods I nd II as obtained from 4 subjects, and in these subjects the mea sodi m lev l of the other subjects for these periods as used for the calculation of percen age filtered sodium excreted. I period III he ndividual plasma sodium level used period II was used for he calculation.

Phosphorus in urine was determined by the method of Ikeda (1931)

Glucose in urine was measured by the method described by Pincator (1966

Ribonuclease was determined by turbi dimetric method (Houck 1957) The standard used as bovine ribonuclease.

α-amino acid-N was assayed by a direct ninhydrin method (Szentirmai et al 1962 Glut m acid was used as the standard

Protein in urine was determined by the biuret method of Goa (1953) with huma albumin as tand ard after precipitation of th proteins with Tsuchiy reagent according Pincator (1962 For qualitativ demonstration Albustix (Ames Co) was used

Concentrates of urine proteins were obtained by ul rafil ration collodion bags (Membranfilter gesellschaft, Gött gen) under reduced pressure

Electrophoresis of urinary proteins. P per electrophoresis was run pH 8 6, using barbital Veronal buffer The separa ion time was 16 hrs th current of 1 mA per strip. Proteins ere stained wi h Amido-Black 10 B and scanner was used for quan itati evaluation

Immunoelectrophoresis Microslid immunoelectrophoresis was ru i principle accordi g to Scheidegger (1955 Horse antiserum against huma serum-protein (N 13189 he Pasteur I stut P ris) was used. Absorbed antiserum was obtained by adding normal urinary protein from period I in the 1963 group

Gel filtration For theory nd pplication see Flodi (1962 *In 1964* separa ions ere done h Sephadex G-100 ol mn, the eluating agent being 0.5 M N Cl 0 1 M sodium phosphate pH 6 8 Column dimension 40 2 5 cm Fiv ml fractions ere collected (f action ollect or eq pment from LKB-produkter AB, Stockholm 12, S eden Concentra ed urinary proteins ere pooled in three groups accord ng to the mount of protein per 1 000 mosm he original ine Subjects 1—4 7—12 nd 13 —19 were pooled. One ml of each as separa ed One ml of an urinary protein oncentrat f om normal man collected a rest, one ml of normal plasma and ne ml of urine oncentrat from ase of ubular proteinuria (chronic adm um poison ng wer also studied. The concentra ed pooled fractions were also investigated by paper elec rophoresis The protein content was determined by he biuret method in the pooled fractions I 3 obtained during gel filtration.

I 1965 separations were done under the same experimental condi ions except tha he elua ng gent was 0.1 M NaCl 0 01 M sodi m phosphate pH 8 6 I wo ubjects separ ions done on the concentrated urinary protein from all three collecting period

Current statistical methods were used The significanc of indi idual differences be een he three urine collecti g periods in the 1965 group was tested by Student test The significa ne expressed as follows

x P<0 05

xx P<0 01

xxx P<0 001

I this calculation one subject (T ble VI) was excluded bec use most of his urinary par meters during the race fell outside 2 SD for the material

TABLE II Excretion of protein, glucose, α-amino acid N and ribonuclease per 1000 mosm and electrophoretic distribution of the urinary proteins t pH 8 6 in the 1964 test group. The subjects are listed in order of increasing mount of protein excretion.

	mg per 1000 mosm				Electrophoretic distribution of proteins t pH 8 6					
Subject	Protein	Glucose	α-amino-acid-N	RNase	Alb.	α_1	α_2		β	γ
1	120	19.8	218	1.03	28.6	—	—	71 4	—	—
2	241	4.3	177	0.69	25.8	12.3	18.4		22.0	21.5
3	245	39 9	256	1.07	36.9	—	—	63.1	—	—
4	248	6 4	181	1.60	30.0	—	—	70.0	—	—
5	266	50.1	266	2.17	25.6	11.2	16.8		26.4	20.0
6	370	70.6	269	1 47	40.3	12.8	16.2		17.5	13.2
7	408	56.5	310	1 74	37.2	9.8	14.0		22.6	16.4
8	411	17.5	252	2.06	39 7	9 1	14.0		21.2	16.0
9	421	62 2	254	1 45	45 7	8.3	15.5		15.5	15.0
10	4 3	100 8	300	1.54	—	—	—		—	—
11	570	50.0	237	1.30	33 4	10.6	12.7		16.3	27 0
12	626	203.4	300	2.20	47.9	4.8	9.2		14 7	23.4
13	649	23 1	282	2.32	42.[illegible]	10.1	18.0		13.3	16.2
14	710	123.7	214	1 57	47.6	6 6	13.9		13.5	18.4
15	727	36.4	[illegible]31	1.82	42.4	13.0	14 1		14 5	16.0
16	735	78.4	284	2.36	43.5	7.5	15.5		12.8	20.9
17	822	27 9	202	1 78	44 2	12.3	17.5		14.5	11.5
18	902	70.5	268	2.00	50 7	10 2	14.2		13.2	11 7
19[a]	1,560	9.5	238	1.[illegible]1	59.2	9.6	11.5		10.8	8.9
20	1,565	47 1	230	1 35	53 7	12 8	11 7		12.8	9 0

[a] This subject, hen checked later showed normal electrophoretic pattern in night specimen, but in day specimen glomerular pattern, of the same type as during the ski race.

Results

Observations at 1964 race

There were large individual variations both in protein excretion and electrophoretic pattern (Table II). Most subjects showed high percentage of albumin. There was a significant correlation (r = 0 83 P<0 001) between protein excreti n and percentage of albumin s measured by electrophoresis. There was no correlation bet een protein excretion and α-amino acid N and ribonuclease excretion (r = 0 09 = 0 02). Between α-amino acid N nd ribonuclease excretion there was a signif icant correlation = 0 72 P<0 001). Fig 1 shows the electrophoretic patterns of the pooled urine of three groups, subjects 1—3 7—12 and 13—20 with low moderat and high protein excretion in the urine. Fig 2 shows the gel filtration pattern of the same three groups and also, for comparison, normal plasma, normal urine protein and urine proteins from one patient w th tubular proteinuria (chronic

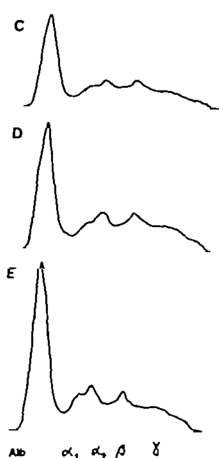


Fig 1

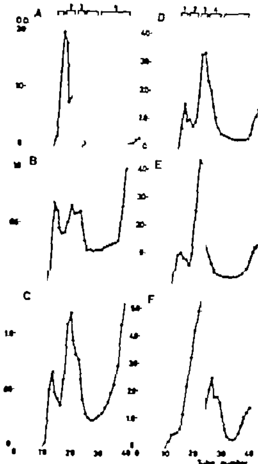


Fig 2

Fig 1 Scanned electrophoretic patterns of pooled urines pH 8 6 of subjects with C) low protein excretion, D moderate protein excretion, E) high protein excretion during ski race
Fig 2 Gel filtration diagrams of A) normal serum proteins, B) normal urine F) urine from patient with chronic cadmium poisoning. C, D E. pooled urine from subjects with low moderate and high protein excretion, respectively during ski race. Scanned electrophoretic patterns of the pooled urine are shown in Fig 1 The absorbancy after fraction 35 refers to non-protein substances

cadmium poisoning) In all three groups there was a considerable increase in fraction no 3 corresponding to serum albumin mol wt ca 69 000 most marked in the group with the highest percentage of albumin

Table III shows that with increasing protein excretion there is a tendency to relative increase in fraction 2 containing mainly γ-globulin and transferrin and a marked increase in fraction 3 composed mainly of albumin There is a relative decrease in fractions 1 and 5 containing respectively the highest and lowest molecular weight proteins. Compared with normal proteinuria, however there is an absolute increase in all fractions, expressed as mg protein per 1 000 mosm In the patient with tubular proteinuria most proteins were found in fractions 4 and 5 containing low molecular weight proteins

TABLE III. The percentage distribution of proteins in the fractions (1—5) collected during gel filtration (Fig 2)

Fraction	Serum	Normal urinary proteins	Pooled urine C	Pooled urine D	Pooled urine E	Tubular proteinuria
1	7.5	10 7	7.3	3 4	3.2	1 1
2	25.1	22.2	10.3	12.6	12.6	5.9
3	65.2	28.2	45.5	51.0	65.1	24.2
4	2.2	27.0	26.6	25 6	15.5	41 4
5	—	11.9	10.5	7 4	3.6	27 4

TABLE IV Mean values, SE and significant difference of plasma parameters measured t start and end of ski race in 1965 test group.

Parameter	Start $\bar{X}$ SE	p[1]	End $\bar{X}$ SE
Plasma creatinine mg/100 ml	1.01±0.05 n = 11		1.07±0 04 n = 12
Plasma potassium meq/l	4.85±0 14 n = 11		4.43±0 15 n = 11
Plasma sodium meq/l	144±2 = 11		146±1 n = 11
Plasma osmolality mosmol/kg H O	784±3 n = 11	XXX	309±2 = 9

Statistical difference (see Methods

Observations at 1965 race

Plasma creatinine P_X P_K *and* P_{osm} (T ble IV) There was no change n plasma cre atinine Plasma sodium concentration increased and potassium decreased slightly Plasma osmolality was significantly increased at the end of the race

Urinary parameter

Table V shows mean alues and SD of all urinary parameters measured during the 1965 race excluding one subject who reacted atypically The ndi idual values of this subject are shown n Tabl VI The significance of ind vidual differences between the three periods is also shown.

Urine flow and renal learance There was a slight decrease in urine flow a somewhat more marked decrease in osmolal nd creatinine clearances and an increase in free water clearance All these changes were not statistically sign ficant. The urine/ plasma creatinine ratio showed no significant change during the race. After the race

TABLE V. Mean values and SD of all measured parameters of the 1965 test group before (Period I) during (Period II) and after (Period III) the ski race. The significant differences between the periods are also shown.

Urinary parameters	Period I Before $\bar{X} \pm SD$	p[1] I—II	Period II During $\bar{X} \pm SD$	p[1] II—III	Period III After $\bar{X} \pm SD$	p[1] I—III
Urine flow ml/min	0.73 ± 0.23		0.50 ± 0.23		0.90 ± 0.57	
Free water clearance	−1.5 ± 0.7		−1.1 ± 0.7		−2.1 ± 1.1	
Osmolal »	2.2 ± 0.8		1.6 ± 0.8		3.0 ± 1.7	
Creatinine	121 ± 30		87 ± 32		107 ± 36	
Urine/plasma creatinine ratio	187 ± 60		191 ± 61		147 ± 5[illegible]	XX
Phosphorus g/g creatinine	0.77 ± 0.23	XX	0.43 ± 0.23	XXX	0.80 ± 27	
Potassium meq/g creatinine	29 ± 14	XXX	74 ± 30		78 ± 34	XX
Sodium meq/g creatinine	71 ± 23	X	40 ± 23	XX	74 ± 37	
Per cent filtered sodium excreted	0.[illegible]0 ± 0.16	X	0.30 ± 0.17	XX	0.64 ± 0.23	
Total protein mg/g creatinine	53 ± 24	XXX	161 ± 76	XX	71 61	
Albumin mg/g creatinine	15 ± 7	XX	68 ± 45	XX	17 14	
Ribonuclease mg/g creatinine	1.01 ± 0.33		0.94 0.38		1.02 0.31	
α-amino acid-N mg/g creatinine	163 ± 51		132 ± 56		174 [illegible]	
Glucose mg/g creatinine	21 ± 11		27 ± 15		[illegible]7 18	
Total protein mg/min	0.075	X	0.1[illegible]6		0.093	

Statistical difference (see methods)
X $P < 0.05$ XX $P < 0.01$ XXX $P < 0.001$

however it was significantly decreased compared with the period of rest before the race. In period I before the race urine flow correlated with urine plasma creatinine ratio but not with creatinine clearances. In the second period during the race the urine flow correlated also with creatinine clearance (Table VII).

Electrolyte excretion. The excretion of phosphorus decreased and of potassium increased, significantly during the race compared with the pre-race values. After the race the excretion of phosphorus, but not that of potassium, returned to pre-exercise level. Sodium excretion and the per cent filtered sodium excreted [illegible] decreased significantly during and returned after the race to pre-exercise level. The sodium excretion during the first and second period was best correlated with the percentage of filtered sodium excreted, but there was no correlation between sodium [illegible] excretion in urine and creatinine clearance (Table VII).

Excretion of protein and low molecular substances. There was a significant increase in albumin and protein excretion in period II compared with period I but no significant change in the excretion of ribonuclease, α-amino acid-N and glucose. After

TABLE VI The individual urinary parameters from one subject excluded from the statistical evaluation of the 1963 test group.

Urinary Parameter	Period I	Period II	Period III
Urine flow	0.60	2.1 +	0.51
C_{H_2O}	−1.0	−0.4 +	−0.8
C_{osm}	1.6	1.7	1.3
C_{cr}	98	32	97
U/P cr	164	25 +	185
P g/g cr ex	1.03	2.2 +	0.68
K	25	723 —	24
Na	32	272 +	32
filtr. Na excr	0.35	1.49 +	0.18
Protein mg/ gr cr exc.	33	175	73
Albumin	12	45	29
Ribonuclease »	0.85	3.47 —	0.81
α-amino acid-N	188	450 +	133
Glucose »	35	120 +	24

+ indicates that the value falls outside 2 SD of the rest of the material (see table V)

TABLE VII Urine flow and urinary sodium excretion correlated with other urinary parameters in periods I and II

Correlation X	Period I Before the race	P	Period II During the race	P
Urine flow — $\frac{U}{P}$ creatinine	−0.71	< 0.01	−0.78	< 0.01
Urine flow — C creatinine	−0.04	> 0.02	−0.72	< 0.01
Na excr — filtr. Na excr	0.73	0.01	0.82	< 0.001
Na excr — C creatinine	0.38	> 0.[illegible]	0.37	> 0.1

the race total protein and albumin excretion returned towards the pre-exercise level. The excretion of ribonuclease α-amino acid N and glucose showed no significant changes.

Electrophoresis and gel filtration Paper electrophoresis for the 1963 group showed the same general trend as for the 1964 group

The electrophoretic and gel filtration patterns of two subjects are shown in Fig 3 and 4 One shows the typical pattern of exercise proteinuria with an increase in albumin, seen both in paper electrophoresis and in gel filtration The other subject shows an atypical pattern with no albumin increase, but instead increase in β

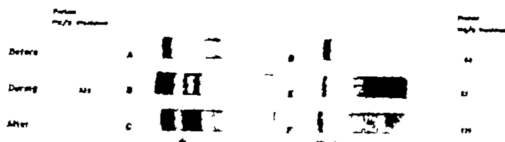


Fig. 3. Original electrophoretic pattern from two subjects before, during and after the ski race. A, B, C typical pattern. D, E, F atypical pattern.

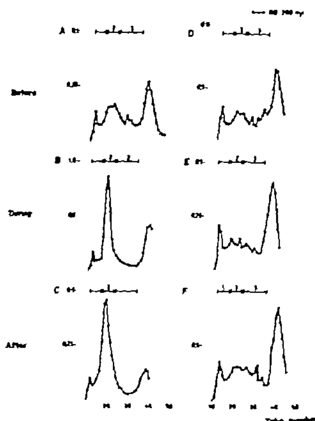


Fig. 4. Gel filtration diagrams from two subjects before, during and after the ski race. A, B, C typical pattern. D, E, F atypical pattern.

and γ fractions (Fig. 3) and a relatively constant gel filtration pattern (Fig. 4). Similar electrophoretic patterns are observed in another 2 subjects, but the rest of the subjects show typical patterns of glomerular type.

Immunoelectrophoresis. Immunoelectrophoretic and immunodiffusion analyses showed in two cases with high protein excretion during exercise that antiserum absorbed with urinary proteins from period I did not react with urinary protein from period II.

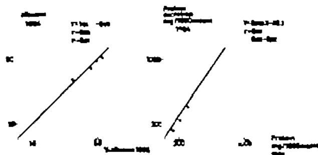


Fig 5 Results btained from 1964 as correlated with the 1965 values for protein excretion and percentage of albumin in 9 subjects.

Comparison of protein excretion 1964 and 1965. Nine subjects participated in the tests both at the 1964 and 1965 races. Fig 5 shows the results obtained from 1964 as correlated with the values of the same individual in 1965 for protein excretion and also for the percentage of albumin. There is a significant correlation between 1964 and 1965

Urinary sediment. During exercise increased amounts of red and white cells and hyaline casts were excreted by most subjects. The Albustix test was slightly positive in three subjects at the end of the race. These subjects also showed the highest protein concentration.

Discussion

The increased excretion of protein in urine associated with exercise may be the result of an increased glomerular load exceeding the rate of tubular reabsorption a decrease in tubular reabsorption without a change in protein filtrations or a combination of both. Urinary proteins may also originate from the lower urinary tract. The urinary TH mucoprotein described by Tamm and Horsfall has been reported to increase during exercise. This protein appears to be of importance in the formation of urinary casts during exercise (Patel 1964) The TH mucoprotein may increase the protein as determined by the biuret method

The increase in albumin excretion found in this study confirms earlier findings (Nedbal and Seliger 1958, Poortmans 1962) and suggests that exercise increases the glomerular permeability for large molecules. Neither immunoelectrophoresis, paper electrophoresis nor gel filtration provided evidence that proteins of high molecular weight not normally found in urine were excreted as a result of exercise.

Alterations in the mechanism for tubula reabsorption of protein are ery dif ficult to measure man. In the present investigation an attempt was made to evalu- te the tubular reabsorptive mechanism by studying the excretion of the low molecula weight protein ribonuclease and amino acids, which may be expected to be almost freely filtrable at the glomerular membrane Changes in urinary excretion of these substances should reflect changes in tubular reabsorption if the filtered load remains the same The excretions of ribonuclease and α-amino acid N did not correlate with the total protein excretion in 1964 and did not significantly

change during the ski race in 1965. This suggests that tubular reabsorption of these substances is unchanged during prolonged exhausting exercise. It may be suggested that this also applies to reabsorption of proteins during exercise. In some subjects, however, as shown in Fig. 3, tubular factors seem to be of importance for exercise proteinuria.

The composition of urinary proteins remained relatively constant from 1961 to 1965 in a given subject (Fig. 5). This suggests that an individual factor in the glomerular membrane contributes to the wide individual variations in the urinary protein excretion in this study. This factor may represent small variations in permeability of the glomerular membrane. The above findings agree with the original hypothesis accounting for exercise proteinuria (Leube 1878). He proposed the theory that individual differences in "der Dichte des Nierenfilters" explain why some subjects increased protein excretion after exercise and some did not. One subject with one of the most marked levels of protein excretion during the race showed typical orthostatic proteinuria at a later check. This suggests that orthostatic mechanisms contributed to the increased protein excretion of some subjects during the ski race.

Most studies of exercise proteinuria (White and Rolf 1948, Taylor 1959, Poortmans and van Kerchove 1962) show that the largest quantities of protein in urine occur after and not during exercise. In this study the protein and albumin excretion increased during exercise and subsided thereafter. This discrepancy may be due to the different types of exercise and shorter duration of collecting period both during and after exercise in the abovementioned studies.

At the 1965 race an attempt was made to evaluate the change in glomerular filtration rate by measuring endogenous creatinine. It was felt that useful information would be obtained although its relationship to glomerular filtration rate is somewhat variable (Smith 1956) and the error of clearance determination is large during periods of low urine flow. The creatinine method used is not specific and may be influenced by chromogens. The constant plasma creatinine concentration during the race suggest that chromogens probably have little influence on the creatinine determinations under these circumstances. The mean decrease in creatinine clearance (28 per cent) is less marked than the decrease in inulin clearance observed in short severe exercise (White and Rolf 1948).

Exercise usually causes a decrease in urine flow, probably due to the liberation of antidiuretic hormone (ADH) and decrease of glomerular filtration rate. The precise contribution of these two factors during exercise has not been evaluated experimentally (Wesson 1960). In this study the decrease in urine flow during the race (−31 per cent) was of the same magnitude as that of creatinine clearance. The urine plasma creatinine ratio, which is a more sensitive indicator of water conservation at low urine flow rate than urine flow itself (Pitts 1963), was not significantly changed. The correlation between urine flow and urine plasma creatinine ratio did not increase significantly from rest to exercise ($r = 0.71$ to $r = 0.78$) but the correlation between urine flow and creatinine clearance increased markedly

(r = +0.04 to r = +0.72). This suggests that the decreased urine flow during the race was to a large extent secondary to decreased glomerular filtration rate.

Muscular exercise is associated with a decrease of sodium excretion in the urine (Bucht *et al.* 1953). This decrease may be due to decreased glomerular filtration or to increased aldosterone secretion or changes in renal hemodynamics. In this investigation there was a decrease of sodium excretion in urine during exercise and also of the percentage of filtered sodium excreted. The excretion of sodium correlated well with the percentage of filtered sodium excreted but not with creatinine clearance before or during the race, suggesting an increased tubular reabsorption. The increased plasma renin activity shown in the 1965 study (Bozović, Castenfors and Piscator 1967) and the increased plasma potassium concentration known to occur during exercise (Laurell and Pernow 1966) suggest an increased aldosterone secretion during the race.

Potassium excretion in the urine during exercise has been reported to decrease slightly or to show no significant change (Kattus *et al.* 1949, Sinclair-Smith *et al.* 1949). The significant increase in potassium excretion both during and after the ski race may have many causes. The increased number of red cells in the urine seen in conjunction with exercise, and also increased hemolysis during exercise may contribute to the increased potassium excretion. Increased aldosterone production may contribute to the potassium loss during the race but probably not afterwards. The rapid increase in sodium excretion following the race is strong evidence against increased aldosterone activity at this time. It is possible that augmented catabolic processes during such long exhausting exercise may result in increased potassium excretion.

The increased excretion in the urinary sediment of red and white cells and hyaline casts is in accordance with earlier findings during exercise (Alyea and Parish 1958, Patel 1964). All subjects in this study increased their urinary protein excretion during the race, only three however reached a level that would be detected by usual clinical tests, showing a slightly positive Albustix test. This illustrates the minute magnitude and physiological nature of exercise proteinuria.

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Renal Clearances and Urinary Sodium and Potassium Excretion during Supine Exercise in Normal Subjects

By

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Abstract

CASTENFORS, J *Renal clearances and urinary sodium and potassium excretion during supine exercise in normal subjects* Acta physiol. scand. 1967 *70* 207—214

Clearance of inulin (C_I) and para-aminohippuric acid (C H) free water clearance (C_{H_2O}) nd urinary excretion of sodium and potassium during exercise were studied in 14 healthy male subjects. The exercise was performed on bicycle ergometer (mean load 729 kpm/min) in the supine position. C decreased significantly but C_{IN} was not significantly changed. The antidiuresis during and after exercise was principally secondary t decreased C_{H_2O}, indicating ADH release The urinary sodium excretion and per cent filtered sodium excreted were significantly decreased during exercise, indicating increased tubular reabsorption of sodium. The findings suggest that he increased tubular reabsorption may in part be due to increased aldosterone ex cretion during exercise Urinary potassium excretion was not significantly changed during exercise

In an earlier investigation Castenf rs and Piscator 1967) th effect of exercise on renal clearances and water excretion was studied t two different w rk loads for the same indi idual. Exercise at heav load was started one hour after the end of exercise t a light load when ll parameters had not returned to the pre-exercise level. Renal electrolyte excret on was not measured.

The present in estigation was undertaken to study the effect of a hea y work load on renal function w thout influence from a previous exercise period. The earlier tud indicated that marked hydration minimizes the effect of exercise on in lin clearance and therefor hea er hydration was used in this study in order better t evaluat t bular effect on renal lectrol t excretion during exercise

Materi l

Fourteen heal hy male olunteers, aged 20—*b* rs, were investigated. All ere well informed about the procedure of he experiment Their ork apacity was determined before the test as the mount of work he subjec ould perform on bic cle ergometer heart rate of 170 beats min

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Methods

Para-aminohippuric acid was determined according to Brun (1951) standard error 1 1 per cent. *Inulin* was determined by the method of Heyrovsky (1956) standard error 0.7 per cent. *Osmolality* was determined by method outlined by Bowman, Trantham and Caulfield (1954) standard error 0 5 per cent or with KNAUER Osmometer 0.5 per cent. *Sodium and potassium* were measured by flame photometry standard error 2.2 and 4.2 per cent respectively *Hematocrit* was determined with an International Centrifuge (11 000 p.m. for 5 min) standard error 1.5 per cent. *Urinary pH* was measured with Beckman pH writer model 72 (corrected for temperature of urine) Standard deviation t pH 5 0 ± 0 027 The pH was determined after the adding of 30—50 ml distilled water used in the bladder-emptying procedure.

The significance of individual differences between the control period (period 2) and periods 3—8 was tested by Student t-test. (x P < 0 05, xx P < 0.01 xxx P < 0.001)

Results

I *Renal hemodynamics* (Fig 1)

C_{PAH} was decreased significantly in all exercise periods and increased again after exercise towards pre-exercise values. C_{PAH} in the third period (5) of exercise was significantly decreased compared with the first period (3) of exercise (P<0 01)

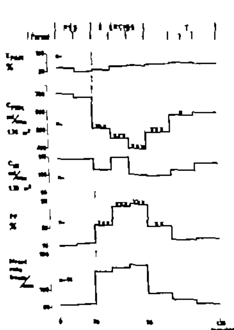


Fig 1



Fig 2.

Fig 1 Effects of exercise on renal hemodynamics. Mean values of E_P C_I FF and heart rate in all periods The statistically significant difference in all periods compared with period 2 is also shown See Methods

Fig 2 Effects of exercise on water diuresis. Mean values of urine plasma inulin ratio, urine flow C ..., C_{osm} C ... ratio in all periods The statistically significant difference in all periods compared h period ... is also shown (See Methods.)

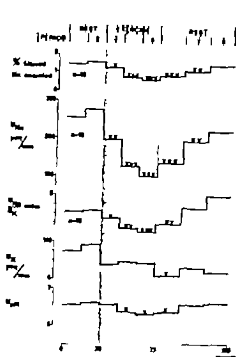


Fig. 3.

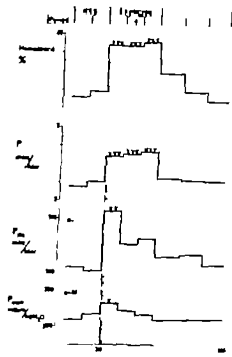


Fig. 4.

Fig. 3. Effect of exercise on urinary electrolyte excretion. Mean values of per cent filtered N excretion (U_{Na}), urinary potassium excretion (L), urinary sodium potassium ratio, and urine pH in all periods. The statistically significant difference in all periods compared with period 2 is also shown. (See Methods.)

Fig. 4. Effect of exercise on hematocrit, plasma sodium (P), potassium (P) and plasma osmolality (P_{osm}). Mean values in all periods are given. The statistically significant difference in all periods compared with period 2 is also shown. (See Methods.)

Extraction of PAH E_{PAH} showed a slight but not significant increase during and after exercise. C_{IN} showed a less marked decrease statistically not significant which was maximal in the first period after exercise thereafter increasing slowly towards pre-exercise level. Filtration fraction (FF) was significantly increased during exercise and in the first period after exercise.

II. *Urine flow and* C_{H_2O} Fig. 2

Urine flow and C_{H_2O} decreased significantly during and after exercise, the maximal decrease occurring in period 7. U_{IN} increased during and after exercise maximally in period 7. The low significance of the changes after exercise in spite of the high mean values was due to wide individual variations in these periods. C_{osm} and C_{osm}/C_{IN} ratio decreased significantly during exercise, maximally in period 5. After exercise they both increased almost to pre-exercise level.

TABLE II Urinary sodium excretion in period 5 and urine flow in period 7 correlated with other urinary parameters.

Independent variable	Dependent variable			P
Urinary sodium excretion Period 5	C_{PAH}	13	0.52	0.05—0.10
	C_{IN}	13	0.26	> 0.50
	Urine flow	13	0.69	< 0.01
	C_{H_2O}	13	0.56	0.02—0.05
	U_K	13	0.88	< 0.001
	% filtered Na excr	13	0.97	< 0.001
	C_{osm}	13	0.85	< 0.001
Urine flow Period 7	C_{IN}	14	0.20	> 0.40
	$\frac{U}{P}IN$	14	0.75	< 0.01
	C_{H_2O}	14	0.97	< 0.001

III *Electrolyte excretion* (Fig. 3)

Urinary sodium excretion and per cent filtered sodium excreted decreased markedly during exercise maximally in period 5. After exercise both increased almost to pre-exercise level. Urinary potassium excretion showed a much less marked decrease than sodium, maximal in the 6th period. Urinary potassium excretion also showed wide individual variations. In period 5 (last period of exercise) 7 subjects had an increased and 6 a decreased potassium excretion compared with period II. The urine sodium/potassium ratio decreased significantly during and increased rapidly after exercise. The urinary pH decreased significantly during exercise (maximal in the last period of exercise) and returned gradually to the pre-exercise level.

IV *Correlation between urinary parameters* (Table II)

During the period of maximal antidiuresis (period 7) the urine flow was significantly correlated to C_{H_2O} and $\frac{U}{P}IN$ but not to C_{IN}. In the last period of exercise (period 5) the urinary sodium excretion showed a highly significant correlation with per cent filtered sodium excreted and with urinary potassium excretion and C_{osm} *but not with* C_{IN}. *Urinary sodium excretion was also significantly correlated* to C_{PAH}, urine flow and C_{H_2O}.

V *Changes in plasma levels* (Fig. 4)

Hematocrit and plasma potassium increased significantly during exercise. After exercise both decreased to pre-exercise level. The decrease in plasma potassium occurred within 1 min of the end of exercise (Fig. 5). Plasma sodium and plasma osmolality increased significantly in the first period of exercise but later showed a gradual decrease during the experiments. Plasma sodium did not decrease to pre-exercise level. Plasma osmolality returned to pre-exercise level in the third period of exercise and decreased even further after exercise.

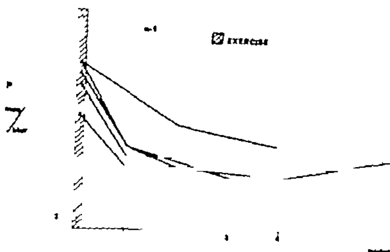


Fig. 5. Decrease in plasma potassium at end of exercise

Discussion

The decrease in C_{PAH} caused by heavy exercise is somewhat less marked, but not significantly different compared with the previous study (Castenfors and Piscator 1967). E_{PAH} was not significantly changed during such heavy exercise indicating that C_{PAH} is a reliable measure of renal plasma flow (RPF) under these experimental conditions. C_{IN} was not significantly decreased which supports the finding in the previous study that marked hydration reduces the effect of exercise on inulin clearance. The results indicate that in man the kidney is capable of maintaining a relatively unchanged glomerular filtration rate during heavy exercise in spite of a substantial decrease in renal plasma flow at least under these experimental conditions. This is probably due to the fact that the afferent arteriolar constriction is balanced by an efferent arteriolar constriction.

Antidiuresis during and after exercise in this study seems to be caused to a large extent by the increased release of antidiuretic hormone (ADH) indicated by the significant decrease in free water clearance (C_{H_2O}) and significant increase in urine plasma inulin ratio ($\frac{U}{P}IN$). The significant correlation between urine flow, $\frac{U}{P}IN$ and C_{H_2O}, but not C_{IN} supports this interpretation. This agrees with the results in the previous study (Castenfors and Piscator 1967).

Hematocrit was significantly increased during exercise (average 6.7 per cent) which agrees with earlier findings and indicates a slight hemoconcentration during exercise.

The significant increase in plasma sodium and osmolality in the first period of exercise is difficult to explain. In the second period of exercise and throughout the whole experiment both parameters gradually decreased, probably secondarily to increased dilution of plasma induced by the oral hydration. The subjects were incapable of inducing water diuresis because of the ADH-releasing effect of exercise

The plasma potassium level was significantly increased during exercise, which agrees with an earlier report (Hultman and Bergström 1962) The increased plasma potassium level decreased within one minute after exercise to pre-exercise level, which agrees with the findings of Laurell and Pernow (1966) that potassium con centration had already decreased to or even below basal level within 30 sec after exercise The increased potassium level probably originates from the working muscle during exercise, secondary to depolarization n the muscle cells or induced by the rapid glucogenolysis accompanying muscl contraction (Bergström and Hultman 1966)

A decrease in sodium excretion during exercise is reported n many studies (Kattus *et al.* 1949 and Bucht *et al* 1953) Decreased urinary sodium excretion can be caused by a decrease in the glomerular filtration rat and/or increased tubular reabsorption of sodium. In the present investigation the d crease in urinary sodium excretion was highly significant and was also significantly correlated to per cent filtered sodium excreted This ndicates that tubular reabsorption plays a domina it role in the decrease of urinary sodium under these experimental conditions The ncreased tubular reabsorption of sodium during exercise may be secondary to changes in medullary blood flow or an aldosterone effect. An increased aldosterone production during exercise has not been reported in th literature, but there are tw indirect lines of evidence suggesting that t does increase First the increased plasma potassium level during exercise found in this study would stimulat an in creased aldosterone production (Davies, Urquhart and Higgins 1963) Secondly plasma renin activ ty is increased during exercise (Helmer 1964 Božović, Castenfors and Piscator 1967) probably causing an increased plasma angiotensin level, which is reported to stimulat aldosterone production (Laragh *et al* 1960 Biron *et al.* 1961) This suggests that aldosterone contributes to the increased tubular reabsorption during xercise. However it is difficult to explain the rapid decrease of sodium excretion during exercise because intra venous dministration of aldosterone is known to ha e a latent period of more than 30 minutes Changes in medullary circulation may probably also contribute to the increased tubular sodi m reabsorption during exercise suggested by the significant correlation between the decrease in C_{PAH} and urinary sodium excretion

The mea urinary potassium excretion was slightly decreased during and after exercise n spi of th ncreased plasma potassium level, though there were wide indi dual ariations In the last period of exercise 7 subjects showed an increase and 6 a decrease i urinary potassium excretion compared with the pre-exercise rate. This agrees w th the results of Kattus *et al* 1949) and S nclair-Smith *et al* 1949 who in about half of their experiments, reported no sign ficant change of potassium excretion nd n other experiments a mall and ariable decrease The effect of exercise on potassium excretion is thus difficult to evaluate. The decrease in urinary pH suggest an ncreased h drogen ion excretion which may compet with the potassium excretion It is possible that the urinary potassium excretion during exerci e is the end result of several opposing factors, competion for excretion

with H decreasing and the suggested aldosterone production stimulating urinary potassium excretion.

Exercise thus causes a marked decrease in sodium excretion and a variable change in potassium excretion. The tubular handling of sodium and potassium during exercise, however seem to be closely related in this study as indicated by their significant correlation in the last period of exercise.

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Acta physiol. scand. 1967. 70. 215—220

From the Department of Clinical Physiology, Karolinska Sjukhuset, Stockholm, Sweden

Effect of Ethacrynic Acid on Plasma Renin Activity during Supine Exercise in Normal Subjects

By

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Abstract

CASTENFORS, J. *Effect of ethacrynic acid on plasma renin activity during supine exercise in normal subjects.* Acta physiol. scand. 1967. *70*. 215—220.

Plasma renin activity, clearances of inulin (C_{IN}) and para-aminohippuric acid (C_{PAH}), free water clearance (C_{H_2O}) and urinary excretion of sodium and potassium were measured in 6 normal subjects at rest and during exercise in the supine position on a bicycle ergometer. The mean work load was 667 kpm/min and the duration of exercise 45 min. Ethacrynic acid, 50 mg, was injected i.v. at the start of exercise. Ethacrynic acid did not influence the normally occurring decrease in C_{PAH} and C_{IN} during exercise but abolished the renal concentration mechanism, indicating an inhibition of sodium reabsorption in the ascending loop of Henle. The urinary sodium excretion increased markedly, indicating an increased load of sodium in distal tubules. This was coincident with increased plasma renin activity. The findings indicate that changes in plasma sodium concentration or sodium load in the distal tubules are of minor importance for the increase in plasma renin activity during exercise.

A previous study (Božović and Castenfors 1967) showed that exercise increased the plasma renin activity and suggested this to be secondary to the increased sympathetic activity irrespective of the renal hemodynamic effect. The decrease in glomerular filtration rate and urinary sodium excretion during exercise suggested that a decreased sodium load in the distal tubules may have been partly responsible for the renin release (Gross, Brunner and Ziegler 1965, Wander 1965).

The present investigation was undertaken to study the effect of increased tubular sodium load on the release of renin during exercise. Ethacrynic acid was chosen because of its rapid and pronounced effect on urinary sodium excretion.

Material, methods and procedure

Six healthy male subjects, age 22—27 years, were investigated. (Table I). Their work capacity was determined prior to the experiment as the amount of work they could perform on a bicycle ergometer at a heart rate of 170 beats/min (W_{170}) (Sjöstrand 1947).

A work load of about 70 per cent of W_{170} was chosen for the study. Exercise was performed in the supine position for 45 min. In the first 5 min of exercise 50 mg of ethacrynic acid (Edecrina MSD) was slowly injected. C_{PAH}, C_{IN}, C_{H_2O}, C_{osm}, urine flow, $\frac{U}{P}_{IN}$, urinary sodium and potassium excretion, plasma sodium and potassium and osmolality were measured before, during and after exercise according to the procedure and the methods described elsewhere (Castenfors 1967).

TABLE I Age, height, weight W_{max} work load, hydration and change in weight of all subjects.

Subj.	Age years	Height cm	Weight kg	W_{max} kpm min	Work load kpm min	% W_{max}	Hydration ml	Change in weight kg
LN	24	174	6[illegible]	1 030	800	76	[illegible]200	
RÖ	22	180	7[illegible]	900	600	67	1,510	−1.3
SL	26	178	79	800	500	63	3,050	−1 1
US	27	1 0	69	1 100	800	73	2,250	±0
CS	22	186	75	800	500	63	2,200	−0 1
MF	22	190	72	1,200	800	67	2 075	−2.7
Mean	24	180	72	975	667	68	2,221	1.0

alue lost.

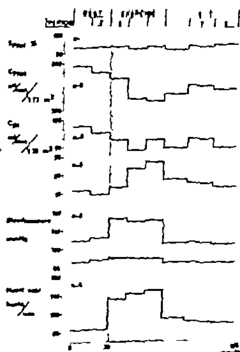


Fig 1



Fig 2

Fig 1 Effect of ethacrynic acid on extraction E and clearance C_P of para-aminohippuric acid, inulin clearance C_I filtration fraction FF blood pressure and heart rate during exercise. Mean values of all periods are given. 50 mg Edecrina injected at the start of exercise.

Fig 2 Effect of ethacrynic acid on hematocrit, plasma osmolality P_{osm} osmolal clearance (C_{osm}) urine flow free water clearance C_{H_2O} and urine plasma ratio ($\frac{U}{P}$IN) during exercise. Mean values of all periods are given. 50 mg Edecrina was injected at the start of exercise.

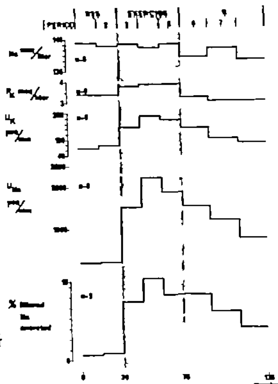


Fig. 3 Effect of ethacrynic acid on plasma sodium (P_{Na}) plasma potassium (P) urinary sodium (U_{Na}) and potassium (U_K) excretion and per cent filtered sodium excreted during exercise Mean alues of all periods are given. 50 mg Edecrina® was injected at the start of exercise

three subjects renal ei catheterization was performed. I one experiment an interruption of the constant infusion of PAH and inulin was not noticed and therefore C_{PAH}, E_{PAH} and C_{IN} could not be calculated Three subjects showed orthostatic hypotension at the end of the experiment, which subsided within 6 hrs

Plasma renin was measured before at the end of and 40 min fter exercise, according to the method described by Boucher *et al.* (1964 with the modification tha heparin was used as anticoagulant instead of EDTA. Plasm rem activ was expressed as the amount of ngiotensin formed af er incubation of plasma for 3 hrs 37 C

Because of he marked effect of ethacrynic acid on sodium excretion in all subjects, the results ere sta istically evaluated only for plasma renin activity They are given as mea alues of all subjects studied

Results

E_{PAH} C_{PAH} C_{IN} FF blood pressure and heart ate

C_{PAH} decreased and E_{PAH} remained unchanged during exercise C_{IN} decreased slightly during and after exercise FF increased during and decreased after exercise Systolic blood pressure ncreased during exercise and decreased after exercise compared w th the pre-exercise pressure Diastolic pressure remained comparatively unchanged during exercise

Hematocrit P_{osm} C_{osm} urine flow C_{H_2O} and $\frac{U}{P}$ IN ratio

The hematocrit ncreased during exercise and thereafter returned slowly to pre-exercise level P_{osm} showed a slight initial increase in th first period of exercise and

TABLE II. Effect of ethacrynic acid on plasma renin activity during exercise

	Subj.	Plasma renin activity ng/angiotensin 100 ml plasma		
		Period 2 rest	Period 5 exercise	Period 8 rest
Arterial plasma	LN	66	1 111	281
	RO	170	256	73
	SL	34	108	80
	US	81	226	96
	CS	119	447	108
	MF	36	205	209
	Mean	*79*	*392*	*161*
	P*		0.05	
Renal venous plasma	LN	204	918	276
	RO	98	256	620
	CS	63	287	282
	Mean	121	481	393

* Wilcoxon Matched-Pairs Signed-Ranks Test

decreased again in the following period, after which no significant changes occurred. C_{osm} increased markedly during and after exercise. Urine flow increased markedly, maximally in the middle of the exercise period, and then gradually decreased. C_{H_2O} decreased during and after exercise. $\frac{U}{P}$IN was unchanged during exercise and increased slightly afterwards.

P_{Na}, P_K, U_K, U_{Na} and per cent filtered sodium excreted

P_{Na} remained unchanged during and decreased after exercise. P_K increased during exercise. U_K increased slightly during and decreased slowly after exercise. U_{Na} and per cent filtered Na excreted increased markedly during exercise and then decreased, but not to pre-exercise level.

Plasma renin activity

The effect of exercise on plasma renin activity is shown in Table II. The plasma renin activity increased during exercise in arterial plasma and renal venous plasma in all subjects. Using the Wilcoxon matched pairs signed ranks test this increase was significant ($P < 0.05$). The post-exercise arterial plasma renin activity decreased to pre-exercise level in 3 subjects. In two subjects it decreased towards pre-exercise level, in one it increased further after exercise. Renin activity in renal venous plasma increased after exercise.

Discussion

Renal plasma flow (C_{PAH}) and glomerular filtration rate (C_{IN}) decreased during exercise as in non medicated normal subjects exercising at comparable work loads (Castenfors 1967). This indicates that ethacrynic acid did not significantly influence the renal plasma flow and glomerular filtration rate which agrees with studies during rest (Cannon, Ames and Laragh 1965).

Blood pressure and heart rate reacted in the same way as in untreated subjects performing almost identical exercise (Božović and Castenfors 1967), which suggests that ethacrynic acid had no major influence on the central hemodynamics.

The increased urine flow during exercise was secondary to increased osmolar clearance, and C_{H_2O} decreased. This is in accordance with earlier reports on the effect of ethacrynic acid during rest (Goldberg *et al.* 1964).

The urine/plasma inulin ratio $\left(\frac{U}{P}IN\right)$ was not significantly changed during exercise, in contrast to non-medicated subjects (Castenfors 1967). This agrees with earlier reports that ethacrynic acid abolishes the urine concentration capacity of the kidney, indicating inhibition of sodium reabsorption in the ascending loop of Henle (Goldberg *et al.* 1964, MacGaffey *et al.* 1964).

Urinary sodium excretion increased markedly, indicating an increased sodium load in the distal tubules during exercise. According to Gross *et al.* (1965) this depress the renin release, but plasma renin activity increased significantly during exercise, even more markedly than in untreated subjects when the urinary sodium excretion decreased during exercise (Božović and Castenfors 1967). The increase in plasma renin activity during exercise, accordingly, does not seem to be attributable to changes in sodium load in the distal tubules.

It has been suggested that the plasma sodium concentration regulates the renin release (Brown *et al.* 1965), high sodium depressing and low sodium stimulating the renin release. In this study plasma sodium was unchanged during exercise, coincident with an increase in plasma renin activity, and decreased after exercise with decreased plasma renin activity. This suggests that changes in plasma sodium concentration are not responsible for renin release during exercise.

Changes in distal tubular fluid composition in close proximity to macula densa have also been suggested as regulating the renin release, an increase in sodium concentration (Thurau 1964) or osmolality (Brown *et al.* 1964) stimulating renin release.

It is difficult to evaluate changes in distal tubular composition. Results from stop-flow experiments indicate that a large dose of ethacrynic acid increased the sodium and potassium concentration throughout the nephron (Beyer *et al.* 1964) and thus also in the distal tubules. If this applies to the present study, the increased plasma renin activity during and after exercise may partly be due to an increase in sodium concentration or osmolality in the distal tubules. However, it is difficult to explain the significant increase in plasma renin activity during exercise in unmedicated and

d hydralazine treated subjects (Bolović and Castenfors 1967) by such a mechanism as it was coincident with decreased sodium excretion.

Accordingly changes in P_{Na} and dramatic changes in renal handling of sodium during exercise did not markedly change the increase in plasma renin activity during exercise. This suggests that the increased plasma renin activity during exercise is caused primarily by a mechanism not related to sodium within or outside the kidney. This is in agreement with the earlier mentioned results (Bolović and Castenfors 1967) which suggests that the increased plasma renin activity during exercise is secondary to the direct sympathetic stimulation on the juxtaglomerular apparatus.

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Recurrent Discharges Studied in Single Units of Laryngeal Muscles

By

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Abstract

Mårtensson A. *Recurrent discharge studied in single units of laryngeal muscles* Acta physiol. scand. 1967 *70* 221—228

I has previously been show tha electrical stimulation pplied to the intac laryngeal muscle nerves of anesthetized dogs elicits, besides the direct response, also secondary centrally relayed response (Mårtensson 1963 The first section of the presen paper describes studies of these responses in single units and provides conclusiv evidence tha he centrally relayed response is not proprioceptiv reflex discharge but represents recurren discharge transmitted through antidromically invaded motoneurons is shown tha when the direct response consists of discharge in single motor unit the secondary response invariably represents discharge in the same unit, nd tha the centrally rela ed response is transmitted by nerv impulses tra elling at the same elocity in the centripetal nd centrifugal direction. In the second section of the paper experimental evidence is presented tha the ecurrent discharges thus recorded in he laryngeal muscles obey the law postulated for the generation of such discharges in spinal motoneurons (Eccles 1955

In a previous nvestigation of the dog laryngeal muscles (Mårtensson 1963 it wa hown that supramaximal stimulation of an intact muscle nerve sets up, in addition to a direct muscle response also a secondary response of low amplitude and longer latency It could be established that the secondary response was of central origin and that both the centripetal and centrifugal olleys were transmitted through the muscle nerve An analysis revealed that the secondary responses did not exhibit properties typical f monosynaptic reflex discharges, and it was suggested that they should instead represent ecurrent d scharges from ntidromically invaded motoneurons (cf. Renshaw 1941 Lloyd 1943)

In this investigation further attempts ha e been made to establish the origin of the secondary responses in the dog laryngeal muscles by means of an analysis of discharges recorded in single units similar to that recently applied by Thorne (1966) on the first dorsal nterosseus muscle in humans. The results to be presented in the first section provide conclusi e evidence that these responses represent recurrent discharges. In the second section it will be shown that the recurrent discharges recorded in the dog intrinsic laryngeal muscles obey the laws postulated for such discharges in spinal motoneurons (Eccles 1955)

nerve. The identical configuration of the responses ind cates that recordings have been made from the same unit. Further support for this could be obtained by combining the stimulations, the stimulus through S following that through S_1 at an interval shorter than the time needed for the impulse conduction along the nerve between the two sites of stimulation As appears from Fig 2 C, no response is then elicited from S_D, indicating that a collision has occurred between the antidromically conducted nerve impulse set up at S_1 and the orthodromically conducted impulse from S_2.

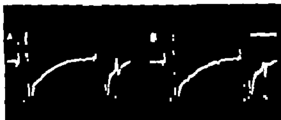
When it had thus been establ hed that the primary responses evoked from the two sites of stimulation represented a discharge in a certain un t, secondary responses were evoked in the same unit by means of double stimuli In Fig 2 D two stimuli are applied through S_1 at an interval of 20 msec, the first generating onl a primary response the second also a secondary response of the same configuration. The two primary responses appear 1 msec after the stimulus artef ct and the secondary response 21 msec after the second stimulus. The long latency is due to the mpulse conduction from S_1 through MN to M In record E tv o stimuli are appl ed through S_2, acti vating the same unit and also in this case the second stimulus sets up a secondary response of the same configuration as that of the two primary ones. In this record the latency of the primary responses is 9 msec (impulse conduction S — M) and that of the secondary response 13 msec (S —MN—M When comparing the latency values of the tv o records it appears that when the stimulus applied through S_1 (record D) the primary response is set up 8 msec (9 minus 1 earlier than when it is applied through S_2 (record E) whereas the secondary response is set up 8 msec (21 minus 13) later Consequently the impulse propagated centrifugally in the motor axon has the same conduction velocity as the centripetal volley centrally activating the motor axon, and the latter should thus represent an antidromically transmitted impulse in the motor axon.

Since the distance from S to the medulla could also be measured in these experiments, the relay time of the centrally relayed responses could also be calculated It was estimated to be about 1 msec which i compatible with the concept of the secondary response as being a recurrent discharge cf Renshaw 1941

II Determinations of stimulus conditions resulting in recurrent discharges

Conclusive evidence having thus been presented that the secondary responses recorded in the dog s laryngeal muscles represent recurrent discharges from antidromically invaded motoneurons, it seemed relevant t study if these responses obey the laws postulated by Eccles (1955 for the generation of recurrent discharges in spinal motoneurons. When applying single ventral root shocks to deafferented animals, Brock, Coombs and Eccles (1953) found that a impulse which propagates antidromically in a motor axon encounters a zone of low safety factor for further transmission at the axon hillock In some cells blockage occurs at this site, a spike recorded intracellularly from the soma-dendrite region bei g then subnormal in size (NM-spike) In other cells the impulse is able to in ade the soma

Fig. 3. Recurrent discharges from two different single motor units in the cricothyroid evoked by application of two successive supramaximal stimuli to its intact muscle nerve. Amplification adjusted so as to show the secondary responses, only the foot of the primary responses being visible. Time bar 10 msec.



dendrite region after a delay of 0 05 to 0 4 msec, and in such cases a fully devel ped action potential (SD spike) is set up, exhibiting a double inflection on the rising phase with a plateau of a duration corresponding to the delay

Eccles assumed that a re-excitation of the axons and a generation of a recurrent impulse would occur on those occasions when the antidromic impulse invades the motoneuron only after a long delay The present investigation has tried to determine the stimulus conditions under which recurrent discharges do actually occur in laryngeal motoneurons, and a comparison of the results thus obtained w th those of Brock *et al.* (1953) might help to clarify the mechanism for the generation of recurrent discharges.

In analogy with the experiments described by Brock *et al* a whole motoneuron pool wa activated antidromically by means of two successive supramaximal stimuli. It then proved to be possible to record recurrent discharges from individual motor units n spite of the maximal stimulation as judged from the finding that recurrent discharges of identical configuration and latency were obtained in several recordings in one and the sam experimental series (see g Fig 4)

In their nvestigations on spinal motoneurons, Brock *et al* found that on single antidromic stimulation some cells were invaded after a brief impulse delay at the axon hillock but others only after a long delay However on pplication of two successive stimuli at a certain nterval there is always considerabl delay before the second impulse invades th soma If the conditi ns for antidromic impulse transmission are the same in the laryngeal as in the spinal motoneurons, t may be expected that while some of the laryngeal motoneurons will be abl to transmit a recurrent response on a single stimulus, others may require double stimulus. That this is actually the case could also be established in the present experiments. When a series of supramaximal doubl stimuli was applied t the intact muscle nerve of the cricothyroid, a secondary response could sometimes be evoked on the first stimulus but it occurred more frequently on the second stimulus. When comparing records from a series of stimulations t could also be established that a considerable number of the recurrent responses elicited by the second timulus represented discharges from motoneurons that were never centrally excited by the first stimulus Fig 3 gives some examples of recording of this type

In a number of such units ttempts were also made to determine the least critical interval required for the elicitatio of a recurrent discharge. B successi ely increasing the stimulus interval from 0 t 150 msec, with several recordings t each single inter

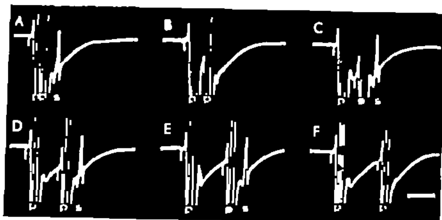


Fig. 4. Recurrent discharges from one and the same motor unit of the cricothyroid evoked by supramaximal double stimulation of its muscle nerve at stimulus intervals of 2.5 (A), 8 (C), 11 (D) and 14 (F) msec. At the intervals of 6.5 (B) and 17 (F) msec no recurrent discharge is evoked in the unit. Each record is obtained by two superimposed sweeps. Time bar 10 msec.

val it could be established that the minimum critical interval varied widely for different units, from 2 msec for several units up to 55 msec for one cell. These values are in good agreement with the least critical interval necessary for the spinal motoneurons to elicit a second spike invading the soma after a long delay at the axon hillock (range from 2.5 to 50 msec).

Brock *et al.* also showed in intracellular recordings of spinal motoneurons that with successive lengthening of the stimulus interval from the least critical value the SD spike exhibited plateaus of long duration over a comparatively wide range of intervals. According to Eccles' hypotheses some motoneurons should transmit a recurrent discharge over a comparatively wide range of intervals, and this was actually a characteristic finding in the present investigation. Thus, while some units were activated over narrow interval ranges, e.g. from 3 to 5 msec, many were activated over a wide range, e.g. from 2 to 20 msec.

For some of the cells in this latter group an intermediate range of intervals was found to be entirely ineffective to elicit a central activation despite a long time of observation. This range of ineffective intervals varied somewhat from one unit to another, a total range from 3 to 9 msec being observed in five different cells. Fig. 4 *A—F* shows recordings from one of these cells. For comparison, Fig. 8 *A* and *B* in the paper by Brock *et al.* illustrates a cell in which the second antidromic invasion occurs after a long axon-soma delay at stimulus intervals between 3.2 and 5.7 msec and also at intervals beyond 9 msec, whereas in the intermediate interval range the second spike only occurred in the shape of an NM spike. According to Eccles' conception no recurrent discharge should in fact be elicited at such intermediate intervals.

Brock *et al.* also observed that if the first impulse invades the soma after a long delay a considerable critical interval is required before the next impulse can invade the soma. Under such circumstances both impulses would actually be able to set up a

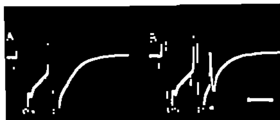


Fig. 5. Primary responses and recurrent discharges from single motor units in the cricothyroid evoked by supramaximal stimulation of its muscle nerve. Stimulus interval 12 msec. When the first stimulus elicits recurrent discharge the second stimulus is either ineffective (A) or evokes recurrent discharge from some other unit (B). Time bar 10 msec.

recurrent discharge according to the hypothesis advanced by Eccles, and the present investigation has substantiated this prediction. Thus, it has been observed that some of the laryngeal motoneurons that were centrally excited by the first stimulus may transmit a recurrent discharge also to the second stimulus when the stimulus interval exceeds 30 msec. At briefer intervals, *e.g.* 12 msec as applied in the recordings in Fig. 5 the second stimulus was either ineffective to elicit a centrally relayed response (A) or evoked a secondary response from some other unit (*B*).

Discussion

When comparing the results obtained in the present investigation with those described by Brock *et al.* (1953) certain differences in the experimental situation must be kept in mind. Thus, in the experiments by Brock *et al.* using ventral root stimulation no proprioceptive afferents were activated, whereas this cannot be definitely excluded in the present study. It is true that the dog laryngeal muscle nerves do not seem to contain any fibers from conventional muscle proprioceptors (Mårtensson 1963), but the possibility must be considered that the nerves may contain afferent fibers from atypical muscle proprioceptors, and if such fibers should have been activated they may have influenced the excitability in the motoneuron pool under study. However, even when making due allowance for these and other possible differences in the two series of experiments it seems to be justifiable to compare the results obtained. As described above such a comparison yields many similarities, and hence the present investigation has undoubtedly provided experimental support for Eccles' conception of recurrent discharges.

The method employed in the present work might also be of some aid for the differentiation between recurrent discharges and reflex responses in animal experiments (Gassel and Wiesendanger 1965) as well as in clinical electrophysiological examinations (*e.g.* Magladery and McDougal 1950, Dawson and Merton 1956).

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The Role of Phosphorylase a and b for the Control of Glycogenolysis in the Isolated Working Rat Heart

By

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Abstract

Øye, I *The role of phosphorylase and b for the control of glycogenolysis in the isolated working rat heart.* Acta physiol. scand 1967 *70* 229—235

Glycogen breakdown and lactate release ha been related to he state of glycogen phosphorylase in the isolated, working rat heart under various experimental conditions. Increased work caused increased ra of glycogen breakdown despite low and constant levels of phosphorylase *a*. In the bsence of added glucose lactate release was small and unaffected by the work load imposed on the heart. Hypoxia caused transient increase in the levels of phosphorylase associated with increased lactate release. This effect of hypoxia was inhibited by th presence of the adrenergic beta receptor blocking agent propranolol and is explained as due t the release of drenergic transmitter from local stores during hypoxia. Prolonged hypoxia and dinitrophenol caused fall in the contractile activity and fall the levels of ATP Under these conditions the hearts showed increased glycogenolysis and lactate release subnormal levels of phosphorylase *a*. This is in accordance with the hypothesis that glycogenolysis under aerobic conditions is restricted by ATP-inhibition of phosphorylase *b* Although glycogenolysis can be regulated at tive withou particip ion of phosphorylase *b* to transformation, the latter mechanism was found be responsible for regulation of glycogenolysis during the early phase of hypoxia. This mechanism must be considered as most significan for the regulation of glycogen breakdow ar ere when the hearts are subject to adrenergic control.

Studies on the kinetics of muscle phosphorylase (α 1 4-glucan orthophosphate glucosyltransferase EC 4 1 1) ha e revealed two basically different mechanisms for control of glycogen breakdown by this enzyme. One is a typical feedback mechanism based on the allosteri acti ation of phosphorylase *b* by 5 —AMP in concentrations w thi the normal physiological range (K_m 0 09 mM) This acti ation is inhibited competitively by ATP and glucose—6—phosphate at physiological concentrations and possibly also by other ell constituents (Morgan and Parmeggiani 1964) The activ ty of phosphorylase *b* in the li ing cell thus depends on the local concentrations of several factors acting on th enzyme The other control mechanism involves reversibl transformation of phosphorylase *b* which is a dimer to phosphorylase *a*, which is tetramer These two nterconvertibl forms of phosphorylase were first discovered by Cori and coworkers Green and Cori 1943 Brown and Cori

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1961) and the transformation process has been extensively studied by Krebs and Fisher (for review see Fisher and Krebs 1960). When phosphorylase *b* is converted to phosphorylase *a* the enzyme is made cofactor independent.

In the present work glycogen breakdown has been studied in relation to the state of phosphorylase under various experimental conditions in the isolated, perfused rat heart. The results confirm that both types of regulatory mechanisms may participate in the control of glycogenolysis in the heart and also indicate that the two basically different mechanisms have distinctly different physiological functions.

Methods

The hearts were perfused by the method previously described (Øye 1965, Hauge and Øye 1966) which is based on the procedure originally developed by Morgan et al. (1963). By this method physiological type of work can be imposed on the heart *in situ*. Rate, pulse pressure, coronary flow rate and cardiac output can be recorded. In the present experiments these parameters were recorded intermittently every 2—5 min during the perfusion period. Hearts which did not reach an adequate and steady physical performance during the initial 10 min in the perfusator were discarded. Adult female breeder rats of local strain were used throughout. The perfusion fluid was Krebs Ringer bicarbonate buffer containing 1 per cent albumin (Bovine, fraction V, Sigma, pH adjusted to 7.4). Perfusions were carried out at 32° in an atmosphere of 95 % O and 5 % CO_2. Were indicated, glucose was included as substrate (final concentration 10 mM).

Samples for lactate determination were withdrawn from the coronary effluent at desired intervals. At end the experiments were terminated by freezing the heart in a Wollenberger clamp cooled in liquid oxygen. Phosphorylase was assayed essentially according to Cornblath et al. (1963). Glycogen was precipitated according to Walaas and Walaas (1950) and assayed by the anthrone method of Carrol et al. (1956). Lactate was assayed enzymatically as described by Hohorst (1963). ATP was assayed by the hexokinase reaction coupled to glucose-6-phosphate dehydrogenase (Lamprecht et al. 1963) and the formation of reduced NADP recorded in a Farrand spectrofluorimeter and compared with NADPH standard read in a Beckman spectrophotometer.

Results

A rapid fall in the heart muscle glycogen concentration occurred during the first minutes of perfusion. The fall was accompanied by release of lactate into the perfusate. In the presence of glucose the glycogen levels were gradually restored during the subsequent perfusion *in vitro*. In the absence of substrates the glycogen

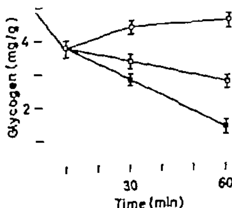
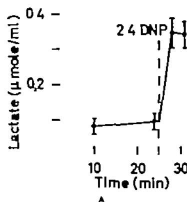


Fig. 1. Glycogen levels in isolated hearts perfused for 10, 30 and 60 min. Experimental conditions as follows: working hearts 10 mM glucose (—○—) working hearts, no substrates (—■—) and non-working hearts, no substrates (—□—). The glycogen level plotted at zero time represent the mean of 8 hearts frozen directly on excision from the animal. The other values are mean values ± one standard error of the mean for 4 hearts.

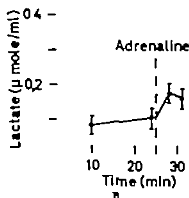
TABLE I Hearts were perfused under standard conditions for 20 min. At this time they were subjected to the experimental procedures listed in the table for 5—15 min. before freezing. Phosphorylase is calculated as the enzyme activity in the absence of AMP expressed as per cent of the activity in the presence of AMP. Two separate samples from each heart were assayed in duplicate and each number is the mean of 5—8 hearts.

Experimental conditions	ATP µmoles/g wet weight	phosphorylase per cent
Control	2.1 ±0.2	7.0 ±0.8
Hypoxia, 5 min	2.0 ±0.3	18.2) ±1.5
Hypoxia, 15 min	0.7 ±0.2	5.0 ±0.9
DNP 1 mM, 5 min	0.5 ±0.2	2.0) ±0.5

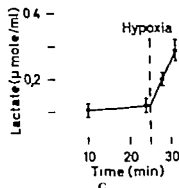
) $p < 0.005$



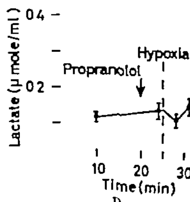
A



B



C



D

Fig. 2. Lactate concentrations in coronary effluent of working hearts perfused in the absence of added substrates. Following control period of 23 min, the hearts were subjected to the experimental procedures listed below.
A. Addition of DNP to final concentration 1 mM.
B. Addition of adrenaline 0.25 µg/ml.
C. Hypoxia obtained by exchanging the gas supply from 95 % O_2 to 95 % N_2.
D. Same as C) but in the presence of propranolol, 2.5 µg/ml added 5 min before hypoxia.

levels continued to decline at a rate which was found to depend on the physical activity of the muscle (Fig 1) Addition of glucose at any time during the perfusion promtly increased lactate output by working as well as nonworking hearts. In the absence of added glucose lactate output was small after the first five minutes No difference in lactate output was found when working and nonworking hearts were compared. The relative amount of phosphorylase *a* was also the same in the two experimental situations (Oye 1967) The increased glycogenolysis during work thus occured without an increase in the levels of phosphorylase *a*, and without an increased release of lactate from the heart.

In order to test the hypothesis that the rate of glycogen breakdown by phosphorylase *b* is restricted by the ATP/AMP ratio under physiological conditions, glycogenolysis was studied in hearts treated with 2 4-dinitrophenol (DNP) or prolonged hypoxia. The addition of DNP to the perfusate caused a slight, transient rise in phosphorylase *a* followed by a fall to levels significantly below those found in hearts perfused without additions. A more extended biphasic response was observed when the hearts were made hypoxic by switching the gas supply from 95 O to 95 N (Table I) In both cases the initial rise in phosphorylase *a* was inhibited by the adrenergic beta receptor blocking agent propranolol (0.25 μg/ml) added to the perfusate 5 min prior to DNP or hypoxia The subsequent fall in phosphorylase *a* coincided with the decline in contractile force and a fall in the concentrations of ATP Glycogen breakdown and lactate output occured at a rapid rate under these conditions, and in the DNP group of heart preparations it was possible to demonstrate rapid lactate release at a time when the levels of phosphorylase *a* were found to be low (Table I and Fig 2A) This was in contrast to the observations made during the first minutes of hypoxia, before the contractile force had started to decline. During this early phase of hypoxia the increase in lactate release was absent when the transformation of phosphorylase *b* to phosphorylase *a* was prevented by pre treatment with propranolol (Fig 2 C and D) The lactate release after adrenaline was somewhat less than the release seen during the early phase of hypoxia (Fig 2 B) A close relationship between the lactate output and the amount of phosphorylase in the *a* form was found on adrenaline administration (Fig 3)

The isolated, working rat heart rapidly adjusts to hypoxia by a decrease in rate and cardiac output (Oye 1967) If adrenaline was added, the hearts maintained high



Fig 3 The relationship between lactate release and the levels of phosphorylase 2 min after administration of 0.03 μg/ml adrenaline to working hearts perfused in the absence of glucose. At this dose adrenaline increased the contractile activity of all hearts, but the effect on phosphorylase was variable. Lactate release in this series was determined from the arteriovenous difference in lactate concentration and the coronary flow rate measured continuously with Palmer photoelectric drop counter

TABLE II. The hearts were perfused under standard conditions for 20—25 min. From this time onward they were subjected to the experimental procedures listed for 5 min, and the perfusion was terminated by freezing the heart as described under methods. Adrenaline was added to final concentration of 0.25 μg/ml. Hypoxia was obtained by switching the gas supply from 95 O to 95 ,, N. DNP was added to final concentration of 1 mM. Glycogen was assayed in tw separate samples from each heart, and the numbers give mean values of 5—7 experiments

Experimental conditions	Glycogen	
	mg/g wet weight	decrease in per cent of control
A. Hearts perfused with glucose 10 mM		
Control	4.37 ± 0.11	
Adrenaline	3.32 ± 0.12	24
Hypoxia	3.09 ± 0.16	29
B. Hearts perfused without glucose		
Control	2.80 ± 0.17	
Adrenaline	2.64 ± 0.17	6
Hypoxia	2.51 ± 0.20	10
DNP	1.71 ± 0.46	39

P 0.01

rate and relatively high cardiac output also during the first minutes of hypoxia, but these hearts were more rapidly exhausted and were found to contain less glycogen than hearts treated with either adrenaline or those ncurring hypoxia alone. Complete depletion of the glycogen stores, however did not occur provided the hearts remained in a functional tate in the sense hat they were able to contract against the hydrostatic pressure of 75 mm mercury which was imposed on the left ventricle in these experiments.

Table II shows the effect of hypoxia and adrenaline on the glycogen stores in hearts perfused with and without glucose As seen from the table, the higher glycogen levels in hearts perfused with glucose as a substrate were more rapidly affected by hypoxia nd adrenaline than the relatively low glycogen levels in the group perfused without substrates. It is oncluded that the rat of glycogen breakdown on hypoxia or adrenalin administration does not only depend on the amount of phosphorylase *a* but also on the stat of th glycogen stores of the heart.

Discussion

It wa shown in a preceding paper that the levels of phosphorylase *a* were not increased when a physiological type of work wa imposed on the isolated, perfused

rat heart (Øye 1967). The results presented above show an increased glycogen breakdown on increased work loads despite the low and constant levels of phosphorylase *a*. Increased glycogen breakdown during increased work took place without increased release of lactate and the rate of glycogenolysis in these hearts thus appears to be coupled to the energy requirements of the muscle by a mechanism that does not cause glycogen breakdown in excess of the amount needed for oxidation. An increase in the oxidative metabolism on increased work loads has been reported previously for this heart preparation (Hauge and Øye 1966).

No significant difference in the steady state concentrations of ATP and glucose 6-phosphate was found when working and nonworking hearts were compared (unpublished observations). The importance of minor changes in the local concentrations of these factors for the regulation of glycogenolysis under various work loads remains unclear. However, following DNP or prolonged hypoxia, low levels of ATP were associated with rapid glycogenolysis despite the very low levels of phosphorylase *a* present under these conditions. This is in accordance with the hypothesis of feedback inhibition of phosphorylase *b* by normal ATP concentrations (Morgan and Parmeggiani 1964).

Conversion of phosphorylase *b* phosphorylase *a* offers an alternative mechanism for increasing the rate of glycogenolysis. Apparently this mechanism is not involved in myocardial autoregulation of glycogenolysis during work and hypoxia in the absence of adrenergic stimulation. It has therefore been proposed that this mechanism is specifically related to adrenergic stimulation (Øye 1967). When phosphorylase *b* is converted to phosphorylase *a*, the enzyme is made cofactor-independent, and the feedback control of glycogenolysis at the phosphorylase level is abolished. The result is glycogen breakdown in excess of the amount needed for oxidative metabolism, and lactate is released from the heart. The physiological significance of breaking down glycogen to lactate under aerobic conditions is not obvious. However relatively large doses of adrenaline are required to trigger this process, and both the inotropic and chronotropic effects can be obtained without increasing the levels of phosphorylase *a* when small doses of adrenaline are administered under aerobic conditions (Mayer *et al.* 1963; Drummond *et al.* 1965). Under hypoxic conditions the effect of adrenaline on the transformation of phosphorylase *b* to phosphorylase *a* appears to be potentiated (Øye 1967). Exogenous adrenaline contributes to the relative hypoxia of the tissue by increasing the oxygen consumption during a state of limited oxygen supply. The potentiation of phosphorylase *b* to *a* transformation might be due to the fact that adrenaline triggers the release of additional amounts of adrenergic transmittors from local stores by increasing the oxygen deficit of myocardium (Wollenberger and Shahab 1965).

In the absence of adrenergic stimulation the isolated heart adjusts to hypoxia by decreased physical activity (Øye 1967). When the ATP/AMP ratio declines, the normal restriction of glycogenolysis by phosphorylase *b* is gradually released. The decreased physical activity as well as the increased glycogenolysis might be regarded as basic homeostatic mechanisms protecting the muscle cells from the effects of

hypoxia. In the intact organisms, on the other hand, a fall in pO in the blood will have to be compensated for by increased, rather than decreased, cardiac work. Concievably adrenergic mechanisms are superimposed on the basic homeostatic mechanisms of the individual cells in order to cope with these requirements *in vivo*

As mentioned above, adrenaline increases the performance of the hypoxic heart. By simultaneously increasing th levels of phosphorylase *a* adrenaline abolishes the normal restriction of glycogenolysis *before* the levels of ATP drop to concentrations incompatible with full contractile activity Thus the two mechanisms of regulation of phosphorylase activity can be regarded as serving basically different physiological functions feedback control of phosphorylase *b* is a cellular autoregulatory mechanism serving the purpose of local homeostasis, while interconversion of phosphorylase *b* to *a* is a specific expression of stimulation mediated through the beta type of adrenergic receptors. The latter mechanism might be of special importance during a transient state of relative oxygen deficit.

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Inhibition of Vagally Stimulated Gastric Secretion after Mucosal Mast Cell Degranulation

By

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Abstract

HIRVONEN J V KAMPPURI and T RÄSÄNEN *Inhibition of vagally stimulated gastric secretion after mucosal mast cell degranulation.* Acta physiol scand 1967 70 236—240.

Total vagotomy was carried out on rats through laparotomy and the peripheral end of the vagus was electrically stimulated for one hour after closing the ventricle with a Shay ligature. After the stimulation, the gastric fluid was collected, its volume, pH and concentration of free HCl were measured. Free HCl was secreted significantly less in the ventricles of rats whose mucosal mast cells had been degranulated by dexamethasone before the vagus stimulation, than in the rats not treated with dexamethasone.

The importance of the synthesis and accumulation of histamine in the mast cells of the gastric mucosa as peripheral link in the stimulatory chain of gastric secretion is discussed.

In the lamina propria of the gastric mucosa there are a very great number of mast cells which react much faster than those in the ileum or the areolar tissue (Räsänen 1960 1963) The action of the mast cells in the gastric mucosa causes, through histamine liberation a stimulation of the parenchymal cells, thus forming possibly a peripheral link in the stimulatory chain whether the stimulus in question is neurogenic or humoral in origin It is possible that the mast cells in the gastric mucosa synthetize a considerable part of the histamine in the whole organism, because the transformation rate of histidine to histamine decreases to one-fourth of normal after gastrectomy (High Shepherd and Woodcock 1963) Histamine disappears from the gastric mucosa during glucocorticoid treatment when the mast cells degranulate (Foley and Glick 1962) At the same time, the stimulation which is normally provoked by Shay ligature seems to be inhibited (Haikonen and Räsänen 1963)

During vagal stimulation histamine is liberated into the gastric fluid Code 1956) The mast cells in the gastric mucosa degranulate faster in insulin hypoglycemia than under the influence of glucocorticoids (Räsänen 1963) During the vagal excitation caused by hypoglycemia the acetylcholine liberated from the neural ends possibly

acts as a histamine liberator and mediates the stimulus to the parenchymal cells. The peripheral link in this stimulatory chain possibly breaks and the gastric secretory response to the vagal stimulation is inhibited if the mucosal mast cells are degranulated, which can be brought about very effectively with dexamethasone (Räsänen 1962).

Material and method

The experiments were performed on 50 male rats of Sprague Dawley strain, weighing 180—230 g, aged about 6 months. Before the experiments, the rats were adapted to normal laboratory conditions for 10—14 days and were given standard commercial pellet food and fresh tap water *ad libitum*. The dry food was exchanged for an amino-acid-glucose-electrolyte mixture 30—36 hours and water was withheld 10 hrs before the operation.

At laparotomy under Nembutal anesthesia both vagus nerves were cut with the aid of a stereomicroscope and electrodes were placed on the peripheral ends which were stimulated electrically with sinusoidal current (12 V, 100 cps). A Shay ligature was tied around the pylorus. The effect of the stimulation was always controlled, and the increased peristalsis in the gastric musculature was followed. The stimulation lasted for one hour only. The control animals were operated on in the same way but were not stimulated. Thereafter the ventricle was removed, dried on the outside and opened. The gastric content was filtered, the volume and pH measured and the free hydrochloric acid titrated with 0.1 N NaOH and methyl red as the indicator.

The mast cells in the gastric mucosa were degranulated with dexamethasone, (Decadron, Merck Sharp & Dohme) given intramuscularly 5 × 1 mg every 12th hr on the days before the operation. The specimens from the gastric mucosa were fixed in four per cent alkaline Pb-acetate. The sections were stained with toluidine blue for mast cells.

Results

The electrical vagus stimulation was performed on 25 rats, of which 13 had been given prior dexamethasone treatment. The volume of the gastric secretion was equal in both groups, but the pH of the gastric fluid from the untreated rats was clearly lower than that of the dexamethasone group ($p < 0.01$, Table I). The concentration of free hydrochloric acid was very significantly higher in the former than in the latter group ($p < 0.001$), in which an almost complete degranulation of the mucosal mast

TABLE I. The acid secretion in the pyloric ligated rat stomach during the electric stimulation of the peripheral end of the cut vagus, and after degranulation of mucosal mast cells with dexamethasone

Treatment	Number of rats	Volume	pH	Free HCl meq/100 ml
Pyloric ligation Vagal stimulation	12	1.4 ± 0.15	1.9 ± 0.09	3.3 ± 0.5
Mast cell degranulation Pyloric ligation Vagal stimulation	13	1.3 ± 0.2	3 ± 0.4	1.0 ± 0.3
Mast cell degranulation Pyloric ligation with [illegible]	13	0.2 ± 0.07	4.0 ± 0.3	0
Pyloric ligation with [illegible]	1	< 0.5	> 4	0

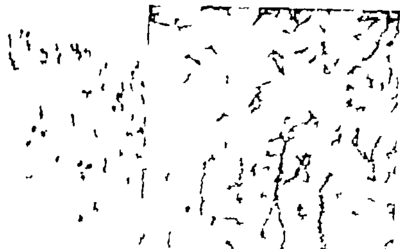


Fig. 1. A. Normal gastric body mucosa of rat rich in mast cells. Magnification 120. B. Loss of the metachromatic coloured granules of mucosal mast cells after dexamethasone treatment. Magnification 450

cells could be demonstrated histologically (Fig. 1). In the nonstimulated animals, with or without dexamethasone (13 and 12 rats, respectively), the gastric secretion was so small that its volume could not be measured. The pH of the gastric content was more than 4 in both non-stimulated groups, and no free HCl was secreted.

Discussion

A relatively short period of vagal stimulation was chosen in order to avoid a major endocrine stimulation effect on secretion. It has been shown in Rhesus monkeys that hypoglycemiainduced stimulation and a decrease in pH occur within one hour of insulin administration, whereas the stimulation of the pituitary-adrenal system causes an increase in gastric secretion only after 3 hrs, also following vagotomy (French *et al.* 1953). This is probably because of the slow conjugation of glucocorticoids compared with the rate of action of neurohormones.

Cortisone can produce an approx. 6-fold increase in the secretion of hydrochloric acid into the sack of Heidenhain after the adrenalectomy without vagal stimulation (Nicoloff *et al.* 1961). The increasing the number and granulation of mucosal mast cells (Räsänen 1961) and more than treble the histamine content in the rat gastric mucosa (Rose and Browne 1940) make the overstimulation with glucocorticoid possible.

Vagotomy almost completely prevents the secretion of free HCl from the ventricle with a pylorus ligature (Shay, Komarov and Gruenstein 1949) as well as in this study. It diminishes the secretory response to the maximal histamine stimulation about 70 per cent (Gelb, Baronofsky and Janowitz 1961) but makes the gastric mucosa hypersensitive to other acetylcholinelike pharmacas (Muren 1959). Vagolytic

treatment also antagonizes effectively the stimulation caused by the exogenic histamine in man (Raju and Nariehvala 1966) and so does the vagal denervation with the humoral stimulation in dog (Olbe 1963)

The mast cells are mostly located in the superficial part of the gastric mucosa where they are exposed to traumas caused by food and peristalsis. It is likely that they transform 5-hydroxytryptophane and histidine to the respective amines, because the blood serotonin concentration falls after partial gastric resection (Schmith, Kinzelmeier and Seng 1959) and the transformation of histidine to histamine is inhibited after gastrectomy (High *et al.* 1963) The gastric secretion induced by histidine is inhibited after mast cell degranulation (Räsänen 1966) Histamine liberation and the secretion in the ventricle with a Shay ligature probably increase in the beginning degranulation phase of mast cells (Haikonen and Räsänen 1965) The stimulatory effect of local histamine liberation can be inhibited with exogenic heparine (Thompson *et al.* 1963) *The inhibition of the effect of vagal electrical stimulation seen in* the rats treated with dexamethasone, possibly arises from the fact that the mucosa contained no active cells to release histamine to mediate the neural as well as the humoral stimulus. The blockage of the cholinergic neural pathway which is the route by which exogenic histamine stimulation is possibly mediated to gastric mucosa (Clark *et al.* 1964) probably occurs between the nerve endings and the mast cells in the gastric mucosa

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The Interaction of Secretin and Pancreozymin on the External Pancreatic Secretion in Dogs

By

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Abstract

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The pancreatic secretion of fluid, bicarbonate and protein in 4 dogs with chronic Thomas type fistulae, has been studied in response to secretin (1 U/kg b.w.), pancreozymin (1 U/kg b.w.) and combined secretin-pancreozymin (1 U/kg b.w. of each). The secretion rates of fluid, bicarbonate and protein after combined, submaximal stimulation were highly reproducible, as indicated by the coefficients of variation 1.7 per cent (fluid), 3.9 per cent (bicarbonate) and 5.1 per cent (protein). The secretion rates of fluid and bicarbonate obtained after the combined stimulation with secretin-pancreozymin (1 U/kg b.w. of each) were very close to those obtained after isolated stimulation with maximal doses of secretin. Secretin and pancreozymin are shown to have a potentiating effect on the pancreatic secretion of bicarbonate, but only an additive effect on the secretion of protein.

The effect on the pancreatic secretion of isolated secretin and pancreozymin has been extensively studied. The effect of combined secretin-pancreozymin stimulation, however, has only been studied to a limited extent. Recently Hansky *et al.* (1964) have used combined, maximal secretin-pancreozymin stimulation in an attempt to establish a maximal pancreatic stimulus.

It has been the aim of the present investigation to study the effect of combined, submaximal doses of secretin and pancreozymin on the external pancreatic secretion in dogs.

Materials and methods

Four mongrel dogs (weight 21.0—28.0 kg) were used. After ligation of the accessory pancreatic duct, a modified Thomas cannula (1941) was inserted into the duodenum opposite the main pancreatic duct. The same type of cannula was inserted into the stomach at the most dependent part. The dogs were used in experiments after a recovery period of at least 3 weeks.

During the experiments the dogs were standing in a sling harness. The gastric cannula was opened to prevent gastric secretion from entering the duodenum. A glass catheter connected to a glass syringe by means of a short polyvinylchloride tube was inserted into the main pancreatic

duct through the open duodenal cannula. The pendent plunger of the syringe exerted slight negative pressure. The dead space of the collecting system was 0.4 ml.

Except for small amounts of water the dogs were fasted 18 hours prior to the experiments. The fasting pancreatic secretion was collected for at least two 15 min periods. After stimulation of the secretion the juice was collected in 10 + 10 + 20 + 20 min.

The stimulants applied were:

1) Secretin (Boots) 1 Unit/kg body weight (b.w.) or

2) Pancreozymin-cholecystokinin (Cecekin, Vitrum) 1 Ivy dog Unit/kg b.w. or

3) Secretin (1 U/kg b.w.) + Pancreozymin-cholecystokinin (1 U/kg b.w.)

As recently shown by Jorpes and Mutt (1966) pancreozymin and cholecystokinin are most likely one single hormone. In the following therefore the term pancreozymin will be used instead of the term pancreozymin-cholecystokinin.

The stimulants were dissolved in isotonic saline to a concentration of 10 U/ml and injected in a peripheral vein in 10—15 sec. Each of the three stimulants were, in randomized order applied three times to each dog. The interval between subsequent injections on the same dog was at least 120 min on account of the "refractory period" of the exocrine pancreas demonstrated after secretin stimulation (Henriksen 1966) and only two injections were given each day of experiment.

The volume of the pancreatic secretion and the concentration of bicarbonate and protein in the samples were measured. The concentration of total CO_2 was determined according to Van Slyke manometric method (1924) calibrated by concomitant titrimetric and manometric estimation on pooled canine pancreatic juice. The concentration of total CO_2 is referred to as bicarbonate. At the actual pH in the pancreatic juice the difference is about 1 per cent. The concentration of protein was estimated spectrophotometrically at 280 nm after appropriate dilution of the samples with Sörensen phosphate buffer pH 6.8. The extinction values were converted to concentrations of protein by means of a factor empirically determined through Kjeldahl analysis on pooled canine pancreatic juice.

The secretory responses were expressed as secretion rate of fluid (f, ml/min), concentration of bicarbonate (meq/l), secretion rate of bicarbonate (b, meq/min) and of protein (p, mg/min) based on the secretion in the first 10 min. after stimulation.

Statistics

In each of the dogs the mean (m) and the standard deviation (SD) of the three experiments with any stimulant, secretin (s), pancreozymin (p) and combined secretin-pancreozymin (s + p) were calculated. Significant differences between SD in the four dogs were not seen. Consequently a common SD for each variable (f, b and p) including all 12 expts. was calculated. According to the stimulant applied these common standard deviations were called SD_s, SD_p and SD_{s+p}.

In each dog the difference (d) between the mean secretion rate after combined secretin-pancreozymin stimulation and the sum of the secretion rates after secretin and pancreozymin respectively was calculated. The standard error of this difference (SE_d) was calculated as

$$SE_d = \sqrt{\frac{SD_s^2 + SD_p^2 + SD_{s+p}^2}{3}}$$

The test of significance of the difference d was done by Student's t = d/SEd, available for each dog. The value t for the group of dogs was calculated as $\sum t/\sqrt{n}$, where t is the sum of the individual t-values and n = 4 (number of dogs). In both cases the degrees of freedom were 8.

Results

Unstimulated secretion

Parameters of the secretion rates in the four dogs are seen in Table I. As all dogs have been used in several experiments in more than a year the number of observations is high. Considerable variation in the secretion rates were seen, especially in the secreted volumes. The coefficient of variation was between 100 and 134 per cent. The variation in the protein secretion rates was considerably smaller with coefficients of variation between 37 and 53 per cent.

TABLE I. The non-stimulated rates of pancreatic secretion of volume (ml/min) bicarbonat (µeq/min) and protein (mg/min)

Dog no.		Volume	Bicarbonate	Protein
1	m	0.06	4.0	2.6
		0—0.40	0 1—19 5	0.4—6.4
		143	40	32
		100.0	89 7	53.1
2	m	0.09	8.7	2 7
		0—0.45	0 7—32.4	0.9—5 7
		120	34	28
		107 7	107 7	47 6
3	m	0 04	3 8	2.0
		0—0.24	0 3—18.5	1 1—3.3
		79	17	8
		116.7	116.7	37 1
4	m	0 07	8.8	2 8
		0—0 42	0.5—32 7	0 6—5 4
		67	24	11
		134.0	102 4	53.9
	m	0 07	6.4	2.6
Total		409	115	79
		120 0	93 1	50 8

*m Mean
Range
Number of observations
Coefficient of variation (SD 100 mean)

Stimulated secretion

Irrespective of the stimulus applied th pancreatic secretion increased bove fasting level w thin 1 min after the stimulation The response after secretin and combined secret n-pancreozymin subsided to basal level in about 30 min After pancreozymin basal level was reached in bout 10 min This is th reason why 10 min secretion is used a the basis for all calculations.

Volume

Values for the secret on rat n th different dogs re given in T ble II Increase in the secretion rate was observed all dogs even after pancreozymin, but the coefficient of variation follow ng thi was rather l gh. Th reproducibil ty was by far the best after combined stimulation w th coefficients of ariation between 0 6 and 2.2 per cent.

In all dogs the secret on rat after combined t mulation exceeded the sum of the secretion rates after secreti and pancreozymi T bl II Fig 1 The d fference was not stat stical significant neither ny of th d gs nor in th group of dogs as a whole ($p < 0.1$)

TABLE II. Secretion rate of fluid (ml/min) in the first 10 min after stimulation with Secretin (1 U/kg b.w.), Pancreozymin (1 U/kg b.w.) or combined Secretin-Pancreozymin (1 U/kg b.w. of each)

Dog no.		Secretin	Pancreozymin	Combined	p
1	m	1.25	0.30	1.65	n.s.
		1.02—1.44	0.23—0.35	1.63—1.69	
		17.1	14.5	2.2	
2	m	1.13	0.33	1.54	n.s.
		0.90—1.34	0.28—0.38	1.52—1.56	
		19.6	13.3	1.4	
	m	1.31	0.31	1.68	n.s.
		1.29—1.34	0.22—0.42	1.67—1.69	
		0	32.5	0.6	
	m	1.38	0.32	1.93	s.
		1.24 1.45	0.26—0.40	1.90—1.97	
		8.0	22.5	1.8	
All		15.0	22.5	1.7	

m Mean of three experiments

r Range

Coefficient of variation (SD 100 mean

*p Probability of difference between combined secretion rate and the sum of secretin and pancreozymin secretion rates

TABLE III. Bicarbonate concentration meq/l in the pancreatic juice the first 10 min after stimulation with Secretin (1 U kg b.w. Pancreozymin (1 U kg b.w.) or combined Secretin-Pancreozymin (1 U kg b.w. of each)

Dog no.		Secretin	Pancreozymin	Combined	p*
1	mQ	120.4	81..	135.3	0.05
		116.3—123.8	76.1—89.0	129.7—143.9	
		3.1	8.3	5.6	
2	m	122.9	101.5	134.6	
		114.2—128.1	91.6—120.9	126.7 142.2	
		6.2	16.6	5.8	
3	m	131.0	90.2	133.8	
		124.1—138.0	76.1—97.9	131.5—13 1	
		5.3	13.6	2.2	
4	m	129.8	106.9	133.4	
		127.8—133.0	100.3—116.5	126.7 138.0	
		2.2	7.9	4.5	
All		4.5	12.3	4.7	0.03

*m Mean of three experiments

Range

Coefficient of variation (SD 100 mean

*p Probability of difference between bicarbonate concentration after combined stimulation and after secretin stimulation.

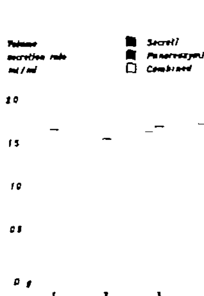


Fig. 1 The pancreatic secretion rates of fluid after submaximal stimulation with secretin (1 U/kg b.w.) pancreozymin (1 U/kg b.w.) and combined secretin-pancreozymin (1 U/kg b.w of each) Each column indicates he mean of 3 expts.

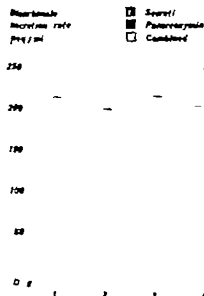


Fig 2 The pancre tic secretion rat of bicarbona after submaximal stimulation with secretin (1 U/kg b.w.) pancreozymin (1 U/kg b.w.) and combined secretin pancreozymin (1 U/kg b w of each) Each column indicates the mea of 3 expts.

TABLE IV Secretion rate of bicarbona (µeq/min.) in the first 10 min after stimulation with Secreti (1 U/kg b) Pancreozymin (1 U/kg b w) or combined Secretin-Pancreozymin (1 U/kg b.w. of each

Dog no		Secretin	P ncreozymin	Combined	p*
1	m	150	24	223	<0 01
		126—173	20—29	215—234	
		16.1	19 7	4 4	
2	m	138	33	208	<0 02
		115—153	26—41	196—216	
		14 5	22 0	5.0	
3	m	171	29	225	n.
		166—178	17—41	219—232	
		3 4	41 1	2.9	
4	m	179	33	258	<0 01
		163—185	30—40	250—266	
		4	15 5	3 2	
All		10 6	26 1	3 9	<0.001

m Mean of three experiment
Range
Coefficient of ariation SD 100 mean
p Probability of differenc between combined secretion rate and he sum of secretin and pancreozymin secretion rates

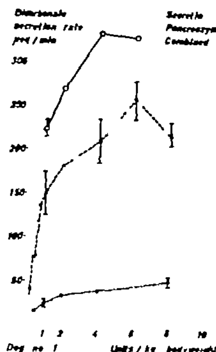


Fig 3. Dose-response curves for secretin pancreozymin and combined secretin-pancreozymin (1 + 1 2 + 2, 4 + 4 6 + 6, U/kg b.w) Vertical bars indicate the range.

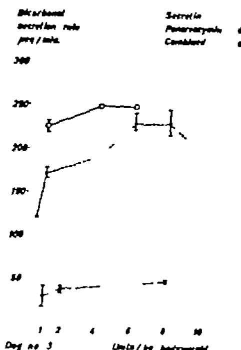


Fig. 4 Dose-response curves for secretin, pancreozymin and combined secretin-pancreozymin (1 + 1 4 + 4 6 + 6 U/kg b w) Vertical bars indicate the range.

Concentration of bicarbonate

Values for the concentration of bicarbonate in the first 10 min secretion after stimulation are given in Table III The concentration increased after stimulation in all dogs irrespective of the stimulant applied The reproducibility was of the same size after secretin as after combined stimulation, but the mean concentration was higher after the latter in all dogs. The difference was significant in one dog and in the group of dogs as a whole

Bicarbonate

The secretion rates of bicarbonate are given in Table IV The secretion rate increased above basal level in all dogs irrespective of the stimulant used, and a remarkable decrease in the coefficient of variation, compared to the unstimulated state (Table I) was observed. Compared with the secretion rate obtained after pancreozymin, the secretion rate after secretin was much higher and concomitantly a decrease in the coefficient of variation was seen. However the secretion rat obtained after combined stimulation was the highest and the reproducibility the best (coefficient of variation 3.9 per cent)

TABLE V Secretion rate of protein (mg/min) in the first 10 min after stimulation with Secretin (1 U/kg b w) Pancreozymin (1 U/kg b.w) or combined Secretin-Pancreozymin (1 U/kg b.w of each)

Dog no.		Secretin	Pancreozymin	Combined	p
1	m	7.6	17.5	23 1	
		4 7—9 4	16 8—18.7	21 7—24.2	s.
		32.2	6.1	5.6	
2	m	10 0	18.2	21 4	
		4.5—12.9	13.3—22.0	21 1—21 7	s.
		49.3	24 4	1 4	
3	m	9 7	19 2	31.9	
		7 4—11.3	15 0—23.6	30.8—32.8	n.s.
		21 4	29 7	3.1	
4	m	5.5	18.2	31 4	
		3.7—8.0	12.5—26.3	29 3—33.5	s.
		41.0	39.5	6.9	
All		38 3	28.1	5.1	n.s.

*m Mean of three experiments
Range
Coefficient of ariation (SD 100/mean)

*p Probability of difference bet een combined secretion rate and the sum of secret and pancreozymin secretion rates.

The mean secretion rate after combined stimulation was higher than the um of the secretion rates after secretin and pancreozymin in all dogs (T ble IV Fig 2) The difference was statistically significant in dog 1 2 and 4 and in the group of dogs as a whole.

In two dogs (no. 1 and 3) the secretion rates after combined stimulation with varying doses of secretin + pancreozymin were investigated. The alues thus obtained ar in Fig 3 nd 4 compared to the dose — response curves for isolated stimulation with secretin and with pancreozymin The response after stimulation was at all dose levels higher than that seen after secretin stimulation and in all experiments but one higher than the sum of the responses after secretin and pancreozymin.

Protein

The secretion rates of protein are hown in T ble V Secretin induced an ncrease in the protein secretion in all dogs, but the reproducibility was rather poor The reproducibility of the pancreozymin — nduced protein secretion was only slightly better After combined umulation the coefficient of ariation was remarkably small er but the secretion rat on an erage did not exeed the sum of the secretion rates after secretin nd pancreozymin

Discussion

The unstimulated pancreatic secretion rates observed here were of the same order of magnitude and with nearly the same variability as those observed by Baron *et al.* (1963) and Hansky *et al.* (1964). Consequently the endogenous stimulation of the pancreatic secretion was nearly identical in the different investigations.

The secretin preparation used here (Secretin Boots) contains about 25 Crick, Harper and Raper units of pancreozymin per 100 units of secretin (information obtained from Boots Pure Drug Company Ltd., Nottingham, England). The pancreozymin preparation used (Cecekin, Vitrum) contains about 6 clinical units of secretin per 75 Ivy dog units of pancreozymin (information from Prof. E. Jorpes and Dr. V. Mutt, Stockholm, Sweden).

The observed increase in bicarbonate concentration after combined secretin-pancreozymin stimulation as compared to the concentration obtained after isolated secretin stimulation (Table III) is not explained by this secretin contamination of the pancreozymin preparation, as the dosage of secretin contained in the pancreozymin administered was less than 0.1 unit per kg b.w. The contamination does not interfere with results obtained for secretion rates, as the responses after combined stimulation in any case was compared to the sum of the responses obtained after secretin and pancreozymin respectively.

The results show that the secretion rates of fluid and bicarbonate after combined stimulation were greater than the sum of the secretion rates after secretin and pancreozymin respectively. The lacking statistical significance of the increase in the secretion rate of fluid ($0.05 < p < 0.1$) may be explained by the limited number of experiments.

In contradistinction to this the secretion rate of protein after combined stimulation was nearly identical to the sum of the secretion rates after secretin and pancreozymin respectively.

In the dogs used in this investigation the maximal secretion rates of fluid, bicarbonate and protein after isolated stimulation with secretin or pancreozymin were obtained with 6—8 units of secretin/kg b.w. or with 12—16 units of pancreozymin/kg b.w. The secretion rates after combined secretin-pancreozymin stimulation (1 unit/kg b.w. of each) were on an average 93 per cent (fluid), 97 per cent (bicarbonate) and 75 per cent (protein) of the maximal secretion rates thus obtained.

Baron *et al.* (1963) and Hansky *et al.* (1964) observed the least variability of pancreatic secretion to be obtained at maximal secretion rates. In the present investigation the variability was least after combined secretin-pancreozymin stimulation with coefficients of variation as low as those observed by Baron *et al.* and Hansky *et al.* after maximal stimulation. The high reproducibility of the secretion of fluid and bicarbonate, therefore, seem to be explained by an almost maximal secretion rate. This explanation, however, seems to be less valid concerning the low variation in the secretion rate of protein.

In accordance with the definition of Goodman and Gilman (1965) secretin and pancreozymin, at a dose level 1 U/kg b.w., exert a potentiating effect on the bicar-

bonate secretion from the pancreas, as the response to combined stimulation exceeded the sum of the responses to secretin and pancreozymin respectively. However Gillespie and Grossman (1964) have pointed out that this definition is not valid if one is working with that part of the dose-response curve where doubling the dose will more than double the response. Dose-response curves for secretin and pancreozymin made for each dog in the present investigation showed that the dose level used (1 U/kg b.w.) was clearly above this part of the dose-response curves. Consequently the potentiating effect is real. This is further substantiated by the fact that the dose response curves for combined stimulation in a considerable dose-range were above the sum of the responses to secretin and pancreozymin respectively (Figs. 3 and 4).

The mechanism of this potentiating effect is quite unknown. It may be suggested that pancreozymin in addition to the effect on the secretion of enzymes, increases the sensitivity of the bicarbonate secreting cells in the gland to secretin.

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Effect of Cholecystokinin on Small Intestine

By

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Abstract

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The effect of highly purified cholecystokinin on gall-bladder and intestine was investigated in vivo and in vitro. The following results were obtained The contraction of small intestine elicited by cholecystokinin preparations is due to the hormone itself and not to impurities in the preparations. The intestinal effect of cholecystokinin is mediated by another mechanism of action than the effect of the hormone on the gall-bladder. Cholecystokinin in low concentrations seems to have a specific effect on the proximal part of the intestine. It is possible that the hormone may play a role in the regulation of intestinal motility

In 1929 Ivy *et al* described the preparation of an extract of intestinal mucosa that contained cholecystokinin activity Jung and Greengard (1933) reported that such extracts caused a contraction of guinea pig ileum in vitro, and Sandblom *et al* (1935) demonstrated an increased duodenal peristalsis in vivo following the intravenous administration of a cholecystokinin preparation. Usually this intestinal effect has been considered to arise from impurities in the cholecystokinin preparations. However the effect has been demonstrated also with more purified preparations (Dahlgren 1964)

In 1964 Jorpes *et al* produced a purified cholecystokinin preparation with an activity of about 3000 Ivy dog units per mg The chemical properties of their preparation suggested that it represented a very high degree of purity It was considered to be of interest to reinvestigate the possible cholecystokinin effect on intestinal motility with this purified preparation.

Material and methods

Sixty-five guinea pigs of either sex, weighing about 200 g, were used in the in vitro experiments About 2 cm long segments of the ileum were immersed into a 25 ml bath containing Krebs solution (37 C), bubbled with a mixture of 95 % O_2 and 5 % CO_2 Isotonic contractions were registered on a kymograph, and isometric contractions by means of a Wheatstone bridge transducer (Swema SG4—45) connected to a potentiometer recorder For in vitro preparation of the gall-bladder

the fundus and collum parts were cut off and the ring thus obtained was cut open to give a strip of gall-bladder tissue which was immersed into 25 ml of Krebs solution.

Ten cats of either sex, weighing 1.8—3.2 kg, anaesthetized with pentobarbitone, were employed for in vivo experiments. Carotid blood pressure, intravesical gall pressure and the pressure in the upper jejunum were simultaneously registered by fluid filled recording systems connected to a Grass polygraph recorder (Model 5 D). A cannula for the registration of gall-bladder pressure was introduced into the common or the cystic duct. The hepatic duct was ligated. The intrajejunal pressure was measured using a polyethene tube, passed through the oral cavity, oesophagus and stomach. The inner diameter of the tube was 1.3 mm. Its distal end was connected to a thin-walled rubber tube which, when in position in the intestine, was filled with water until a suitable pressure was reached.

The purified cholecystokinin preparation used contained 4000 Ivy dog units per mg.* The potency of the crude preparation (Cecekin, Vitrum A.B. Stockholm) was 200 units per mg.

Results

I. In vitro experiments

a) Guinea pig ileum. The purified cholecystokinin caused a contraction of the guinea pig ileum which, in the dose range used, was proportional to the dose administered. There was a typical delay period before the response appeared. Thus the contraction occurred up to 10 sec. later than that seen after acetylcholine. After the lag period the tonus of the preparation increased rapidly and often a series of rhythmic contractions were seen (Fig. 1). The latter could be observed irrespective whether the contractions were registered isometrically or isotonically. As a rule, the response obtained after the administration of 4 units of cholecystokinin = 1 μg was equal to, or greater than, that of 1 μg acetylcholine chloride, corresponding to a concentration in the bath of 0.04 μg per ml. Equal or greater doses of other protein hormones such as ACTH and insulin had no effect on the preparation, nor had human plasma.

Atropine in doses of about 0.1 μg completely inhibited the responses of 1 μg acetylcholine chloride and of 4 units of cholecystokinin. Lower concentrations of atropine caused a reduction of the responses to the two stimulants.

Hexamethonium in doses up to 100 μg did not affect the response to cholecystokinin.

Cholecystokinin had no effect on guinea pig colon or uterus, when investigated by the same technique as that used for the ileum preparations.



Fig. 1. Contractions of guinea pig ileum in vitro registered isometrically. The following substances were administered: A. 1 μg acetylcholine. B. 1 μg histamine. C. 3 μg serotonin. D. 3 units of cholecystokinin (4000 units per mg).

* Kindly supplied by Dr. V. Mutt, Stockholm.

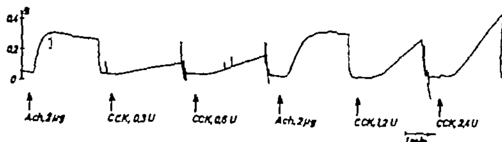


Fig 2 Contractions of guinea pig gall bladder in vitro, registered isometrically Ach stands for acetylcholine CCK for cholecystokinin. With increasing doses of cholecystokinin steeper slope of the response line is seen.

A comparison between two cholecystokinin preparations was made using the response of the guinea pig ileum as the parameter The substances tested were the purified cholecystokinin of Jorpes *et al* (1964) containing 4000 units per mg and the commercially available preparation Cecekin, containing 200 units per mg It was found that the activity of the purified preparation was 21 8 times greater per mg than that of the crude one. The activities of the two preparations were however about the same (109) when compared on an Ivy dog unit basis.

b) Gall-bladder The guinea pig gall-bladder responded to cholecystokinin in lower doses than those required to get a contraction of the ileum As a rule the lowest effective dose was 0 3 Ivy units corresponding to a concentration of 0 003 µg per ml in the medium Even in these experiments a delay of the response to cholecystokinin was seen (Fig 2) With increasing doses the lag period decreased. The response developed uniformly with time The tangent for the angle between the base line and the response line was found to be rectilinearly related to the logdose of cholecystokinin. An example is given in Fig 2

Atropine in a dose of 0.2 µg which inhibited the response to 1 µg acetylcholine, left the response to 0 6 units of cholecystokinin unaffected. No reduction of the response to cholecystokinin could be observed even after an increase of the atropine dose to 50 µg

II In vivo experiments

Blood pressure, gall bladder pressure and intraluminal pressure in the jejunum were simultaneously recorded in the anaesthetized cats. After 2 3—5 units of cholecystokinin intravenously (4000 Ivy units per mg) the gall-bladder pressure increased from about 2 mm Hg to about 10 mm Hg The pressure in the jejunum usually did not exceed 15 mm Hg in the control period After cholecystokinin the basic tonus was elevated about 10 mm Hg above the control level, and there were frequent pressure peaks of short duration up to 50 mm Hg (Fig 3) When the crude preparation of cholecystokinin was used, the blood pressure remained unaffected. With purified cholecystokinin an increase of the blood pressure was sometimes seen

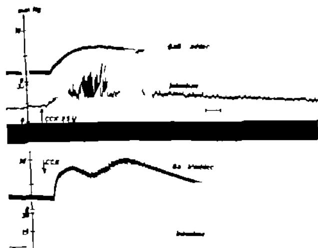


Fig. 3 Effect of cholecystokinin (CCK) on the pressure in gall-bladder and jejunum in cat, weighing 1.9 kg before (upper part) and after (lower part) 3 mg atropine intravenously

After treatment with atropine, 2 mg per kg b w the intestinal response to chole cystokinin was inhib ted but the response of the gall-bladder remained completely unaffected.

Discussion

Highly purified cholecystokinin (Jorpes *et al.* 1964) has been shown to cause a con traction of the small intestine as shown in vitro (guinea pig) and in vivo (cat) The comparison of crude and purified cholecystokinin showed that th intestinal effect was bound to the cholecystokinin activ ty and not to the amount of substance given. These experiments thus provide support for the view that the effect on the intestine is due to the hormone itself and not to impurities in the preparation. This is in accordance with results reported by Dahlgren (1966) who, using a prepara tion of the sam high purity found an increased duodenal peristalsis in the dog

The slow development of the response f the gall-bladder to cholecystokinin observed in the v tro experiments, contrasts to the rapid one in the ileum preparations. This indicates a fundamental difference between the actions of cholecystokinin on the two organs. Furthermore the effect of cholecystokinin on the intestine was inhibited by atropine in low doses This is in contrast to the effect on the gall-bladder which was unaffected also by high doses of tropine Thus t seems reasonabl to assume a different mechanism of action for the effect on the ntestine compared to that on the gall-bladder Valto *et al* 1963) were of the same opinion, but, since they used a crude preparation, non-specific effects of impurities could not be excluded.

When tested in vitro equal doses of cholecystokinin and acetylcholine chloride caused responses of about the same size in the ileum preparation. Consequently the molar concentration of cholecystokinin must ha been lower than that of acetyl-

choline. As the response was elicited by such low doses it is less probable that the effect of cholecystokinin on guinea pig ileum in vitro represents a non-specific protein effect. Furthermore, equal or greater amounts of other protein hormones and of human plasma were inactive. Also in the cat experiments, low doses of cholecystokinin were used, and a calculated plasma concentration of 0.01 μg per ml was active. These small amounts of the hormone being active on the small intestine, may suggest a physiological role for cholecystokinin in the regulation of intestinal motility.

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Lactate and Pyruvate Concentrations in Blood, Cerebrospinal Fluid and Brain Tissue of the Cat

By

LARS GRANHOLM and BO K SIESJO

Interest in the lactat and pyruvate concentrations of tissues and tissue fluids is partly due to the fact that the lactate/pyruvate ratios, or the excess lactate" concentrations, ha e been claimed to indicate tissue hypoxia (Huckabee 1958 Hohorst 1960) The eason for this is the coupling between the NADH/NAD and the lactatate/pyruvate systems nd, since both lactic and pyruvic acid can diffuse freely in tissues, it has been proposed that extracellular lactate/pyruvate ratios can be used as a measure of the redox state of th intracellular NADH/NAD+ systems (Hohorst *et al* 1965) However although attempts ha e been made to correlat lactate/pyruvate ratio n the cerebrospinal fluid (CSF) to various neurological disorders (Kurze *et al* 1966) there seems to be no study of how this ratio i related to the corresponding lactat /pyruvate ratio in brain tissue. In the present paper a report is therefore given of measurements of lactate and pyruvate concentrations n arterial blood, CFS and brain tissue of anesthetized cats.

Spontaneously breathing cats were anesthetized with Phenobarbital (125—150 mg/kg) i p The femoral artery was cannulated for blood pressure recording and for sampling of blood, and the femoral ein for injections. The arterial pH and P_{CO} were measured with micro electrodes CSF was sampled from the suboccipital cistern after exposure of the tlanto-occipital membrane (see Pontén and Siesjö 1966) The dura wa exposed over the pa etal cortices. For the measurements of lactat and pyruvat concentrations blood and CSF were sampled directly into liquid nitrogen. Brain tissue was frozen *in situ* by pouring liquid nitrogen on the exposed dura and the subdural cortical tissue wa chiselled out for analysis. The lactate and pyruvat concentrations were measured w th specific enzymatic methods after homogenization and entrifugation in th c ld neutralization of the perchloric acid ex tract (pH 4.5) with precipit tion of potassium perchlorate, nd ubsequent recentrifugation (Hohorst, Kreutz and Bucher 1959

The esults are gi en h t bl The acid base alues gi en refer to 16 expts with an arterial P_{CO} f between 30 nd 40 mm Hg Measurements of lactate nd Pyruvat concentrations wer made on 15 blood 10 CSF and 7 brain samples. Both the lactat and pyruvat oncentrations n the CSF were larger than those in th

TABLE 1 Acid-base parameters and lactat pyruvate concentrations nd ratios in arterial blood, CSF and brai tissu of nesthetized cats. Means ± S E.M The plasma bicarbonate concentrations were calculated from the pH nd the P_{CO_2} values. The lactat and pyruvate concentrations are expressed per kg of w ter

	pH	P_{CO_2} mm Hg	HCO meq/l	Lactate mmoles/kg	Pyruvat H O	Lactate/ Pyruvate
Arterial blood	7.368	35.6	19.9	0 66	0 073	9 4
	±0 01	±0 7	±0 7	±0 07	±0 005	±0.7
Brain tissue				0.72	0.063	11.5
				±0.03	±0.004	±0.7
CSF				1 16	0.192	6 1
				±0.0[illegible]	±0.009	±0 3

blood or the tissue In addition, the lactate pyruvate ratios n the CSF were lower than those in the blood and much lower than those in the tissue. If the whole blood concentrations are converted into plasma alues (Huckabee 1956) the ratios approach those for CSF but any similarity may be fortuitous since the blood values show a much larger spread, probably due to a ariable operative trauma. The important result, however is that both the lactate pyruvate concentrations and the lactate/ pyruvate ratios of CSF markedly deviate from the corresponding values for the tissue water It is evident that further work is needed on the lactate pyruvate rela tions between extra and intracellular fluids in the brain before CSF measurements can be used as a tool for studying redox states in the tissue proper

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Myogenic Response of Resistance Vessels and Precapillary Sphincters in Skeletal Muscle during Exercise

By

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Abstract

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Capillary filtration coefficient (CFC), reflecting precapillary sphincter tone, and venous outflow of cat lower leg muscle were observed at various transmural pressures before and during muscle work. Increasing transmural pressure in resting muscle 30 mm Hg caused a 35 per cent decrease in CFC, indicating increased tone of precapillary sphincters and, on the average, no change in blood flow, indicating that the increased active tone of resistance vessels closely balanced passive distention. During exercise at 1 twitch/sec, elevating transmural pressure decreased CFC by 50 per cent and increased flow 38 per cent indicating that while precapillary sphincters were still responsive, metabolic demand caused the resistance vessels to behave passively. At 4 twitches/sec the active response of the precapillary sphincters was present, although less marked, whereas the passive behaviour of the resistance vessels was even more pronounced than at 1 twitch/sec. Myogenic autoregulation of filtration exchange via alteration of precapillary sphincter tone may be an important factor preventing gross edema in the legs of upright man during exercise.

The two mechanisms predominantly responsible for the local control of nutritional blood flow of skeletal muscle are related to changes in the metabolic state of the tissue and in vascular transmural pressure.

The metabolic factor was first emphasized by Gaskell in 1877, who observed an increased muscle blood flow associated with tetanic contraction. Many studies have since confirmed and extended his observations on the resistance vessels of exercising skeletal muscle (see Barcroft 1963). Krogh, in 1922, demonstrated that the number of blood-containing capillaries per unit area increases during muscle work. Cobbold *et al.* (1963) and Kjellmer (1964) have demonstrated that the capillary filtration coefficient (CFC), reflecting capillary surface area, may increase 2.5 to 3.0-fold during exercise. This result agrees with the work of Renkin and Rosell (1962) who measured A-V extraction of Rb^{86} and of K during exercise and found a 2.0 to

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2.5-fold increase in capillary transport. These changes in CFC and in capillary transport capacity probably reflect alterations in capillary surface area as determined by precapillary sphincter tone, because there seems to be no increase in capillary permeability associated with muscle exercise (Arturson and Kjellmer 1964)

Bayliss (1902) proposed the concept that a myogenic response to transmural pressure is important for the local control of blood flow That is, increased transmural pressure causes increased resistance to blood flow and *vice versa* Recently the idea that vascular smooth muscle of resistance vessels can respond to stretch and thus regulate blood flow has received wide support (Folkow 1964) Mellander *et al.* (1964) have demonstrated that in both man and cats elevated transmural pressure also results in contraction of precapillary sphincters and thus decreased capillary surface area. This response may be an important factor guarding against formation of edema in vascular beds exposed to high hydrostatic load.

Myogenic and metabolic factors may sometimes cooperate to determine the tone of vascular smooth muscle, as, for example, in situations of reduced perfusion pressure and reduced nutritional blood flow In other circumstances, these factors may compete, for example in the dependent limbs of man during exercise. The work of Cobbold *et al.* (1963) and Kjellmer (1965) showing that the metabolic demand of exercise overrides vasoconstrictor influence of the sympathetic nerves, especially on precapillary sphincters, might suggest that the metabolic effect of exercise would also win over the myogenic constrictor response during elevated transmural pressure There appear to be no data explicitly dealing with this subject. Although it is obvious that an increased blood flow and capillary surface area are beneficial in meeting the metabolic needs of exercising muscle a large capillary surface area in the presence of increased hydrostatic load could be detrimental leading to gross edema. This is expecially relevant when considering the circulation of the legs of man during exercise in upright posture.

Since we know from common experience that exercise in the erect position does not lead to gross dependent edema, there must be mechanisms which mitigate the formation of edema. Our calculations, using data from the literature indicated to us that the "muscle pump" increased tissue pressure, and increased lymphatic drainage would not be sufficient to prevent excessive filtration of capillary *surface area were to 3 times the resting level.*

This led us to consider the possibility that elevated transmural pressure could result in closure of precapillary sphincters and decreased capillary surface area even in the presence of strong vasodilator factors related to exercise Therefore the aim of the present study was to determine the integrated response of resistance and precapillary sphincter vessels to the opposing effects of high metabolic demand and elevated transmural pressure. A preliminary report of this work has been published elsewhere (Lundvall Mellander and Sparks 1966)

Methods

General considerations. The use of a plethysmograph for the measurement of net transcapillary fluid shifts across the vascular bed has been described previously (Mellander 1960 Changes in the

size of the capillary surface area and thus, changes in the tone of the precapillary sphincters, were followed by determining the CFC (Cobbold *et al.* 1963). CFC was determined by raising the venous outflow pressure a known amount and recording the rate of outward filtration caused by the consequent increase in capillary hydrostatic pressure (see Pappenheimer and Soto-Rivera 1948, Mellander 1960). Because calculation of the shift in capillary hydrostatic pressure requires knowledge of the precapillary/postcapillary resistance ratio (Pappenheimer and Soto-Rivera 1948, Cobbold *et al.* 1963), this was determined during work and at high and low transmural pressure. In most cases, the ratio was approximately 4/1 and this value was used for all calculations. During work and at low transmural pressure, the value was occasionally less than 4/1 and thus, CFC during these states may be underestimated, while the value at high transmural pressure during rest was sometimes as great as 5/1 so that these CFCs may be overestimated (see Mellander *et al.* 1964). CFC is expressed as ml fluid filtered per minute per 100 g muscle per mm Hg increase in mean capillary hydrostatic pressure (ml/min × 100 g × mm Hg). If tissue pressure is significant CFC might be underestimated due to the so-called waterfall phenomenon. This factor was taken into account in the present study (see Kjellmer 1964b).

Experimental procedure. The experiments were performed on the lower leg muscles of 15 cats anesthetized with chloralose (50 mg/kg) and in most cases urethane (100 mg/kg). The lower leg muscle preparation has been described in detail by Kjellmer (1964a). The skin was separated from the calf muscle so that it had no blood supply but covered the muscle. The paw was removed and, using the skin for water-proof closure, the lower leg was placed in a water-filled plethysmograph permitting continuous recording of changes in tissue volume. After heparinization, the popliteal vein (the sole venous drainage in this preparation) was cannulated, and venous outflow was recorded continuously using a drop recorder and the blood returned to the animal via a funnel into the femoral or jugular vein. Arterial inflow pressure was controlled by means of a clamp on the abdominal aorta and measured in the femoral artery of the opposite limb. Venous outflow pressure could be set at any level by adjusting the height of the orifice of the outflow tubing. CFC was determined at intervals during the course of the experiment.

Muscle exercise was produced by electrical stimulation of the peripheral end of the severed sciatic nerve at 1 or 4 imp/sec. Supramaximal stimuli (2—3 V, 0.1 msec) known not to excite sympathetic fibres (Kjellmer 1964a) were delivered by a Grass stimulator model S4E. In 5 of the experiments, isometric tension development was measured by means of a Grass FT 10 force transducer attached to the Achilles tendon (Sonnenschein, Wright and Mellander 1967).

Transmural pressure was varied approximately 30 mm Hg by changing arterial inflow pressure and venous outflow pressure the same amount, thus keeping perfusion pressure constant. CFC was first determined at low and high transmural pressure without muscle exercise. After this, the sciatic nerve was stimulated at 1 imp/sec and CFC again determined at the two pressure levels. The same experimental manoeuvres were repeated in some experiments during exercise at 4 imp/sec.

In two experiments, in order to vary the temperature of the arterial blood, the popliteal artery was also cannulated, and the calf muscle was perfused from the carotid artery via polyvinyl chloride tubing. The temperature of the blood was controlled by placing the tubing in a water bath and was measured using a thermocouple inserted into the blood stream. Essentially the same experimental manoeuvres as described above were then performed.

Results

Technically satisfactory experiments involving CFC measurements were performed on 14 cats. In 1 cat only flow was measured. At the beginning of each experiment, CFC was determined at low and high transmural pressure in the resting muscle. Twelve of the 14 preparations showed the typical myogenic response pattern of the precapillary sphincters, i.e. decreased CFC with elevated transmural pressure, but in 2 experiments alterations of transmural pressure produced no significant change in CFC. Findings to support the idea that the decreased reactivity of precapillary sphincters in these two experiments was due to the low temperature of the blood perfusing the calf muscle will be presented below.

The experimentally induced changes in transmural pressure averaged roughly 30 mm Hg. Values for arterial inflow pressure during low transmural pressure periods

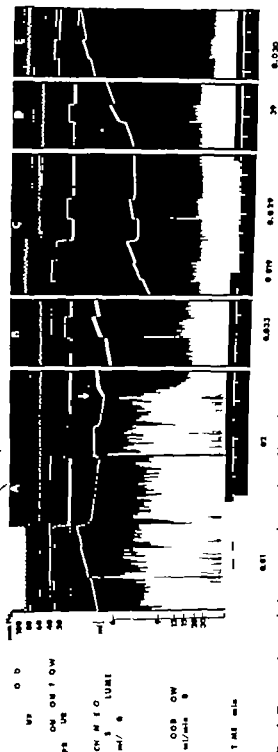


Fig. 1 Kymograph record showing vascular reactions of lower leg muscle preparation to changes in transmural pressure (30 mm Hg) during rest (A) and exercise, first at 1 twitch/sec (B and C) and then at 4 twitches/sec (D and E)

varied between 60 and 95 with an average value of 80 mm Hg while venous pressure was less than 5 mm Hg The calculated mean capillary pressure (Pappenheimer and Soto-Rivera 1948) varied between 15 and 25 mm Hg with 18 mm Hg as the average for all experiments. This gives an indication of precapillary sphincter pressure, which must be slightly higher At elevated transmural pressure arterial blood pressure varied between 90 and 125 mm Hg with an average of 105 mm Hg Venous pressure was raised 25 to 35 mm Hg and thus mean capillary pressure varied approximately between 35 and 55 mm Hg with an average of 48 mm Hg

Fig 1 shows a representative experiment in which arterial inflow pressure venous outflow pressure, blood flow and volume changes of the lower leg muscle preparation were recorded continuously CFC was determined at intervals. The first part of panel A shows the flow and CFC during rest and a high transmural pressure. Arterial inflow pressure was 90 and venous pressure was 33 mm Hg, with a resultant mean capillary pressure of approximately 44 mm Hg The CFC was 0 017 ml/min × 100 g × mm Hg and flow about 8 ml/min × 100 g Arterial and venous pressures were then lowered to 60 and 5 mm Hg respectively and mean capillary pressure was calculated to be approximately 14 mm Hg CFC increased to 0 024 Flow remained essentially the same in spite of the decreased distension of the resistance vessels which must have resulted from lowered transmural pressure Thus, the elastic recoil must have been balanced by relaxation of the vascular smooth muscle

The onset of exercise is marked by the arrow in panel A where the sciatic nerve was stimulated at 1 imp/sec It can be seen that blood flow increased approximately twofold to 16 ml/min 100 g The volume curve shows the typical change during exercise, i e first an abrupt increase due to distension of capacitance vessels and then slower rise which at a given transmural pressure tends to level off with time This latter change is due to transfer of fluid across the capillary membranes related to a rise in hydrostatic capillary pressure (Kjellmer 1964a Panel B shows the records after 2 min interval (volume recorder pen repositioned) when blood flow had reached a steady state CFC determined in this period of low transmural pressure was 0 033 This increase of CFC reflects an enlarged capillary surface area in response to exercise due to relaxation of precapillary sphincters Transmural pressure was then elevated 30 mm Hg by raising arterial pressure to 90 mm Hg and venous pressure to 33 mm Hg panel C The CFC decreased to 0 019 indicating an active constrictor response of the precapillary sphincters to the increased transmural pressure Flow on the other hand increased from 16 to 20 ml min 100 g which reflects a passive behaviour of the resistance vessels The volume curve is somewhat steeper in this phase than B indicating an increased net filtration CFC was then determined during another period of low transmural pressure last part of panel C) but here the perfusion pressure was slightly increased + 5 mm Hg in order to keep blood flow at exactly the same level as in the preceding period of high transmural pressure CFC was 0 0[illegible] showing that even when blood flow was kept constant CFC was significantly smaller at high than low transmural pressure

In the interval between panels C and D perfusion pressure and hence blood flow

was restored to the previous level (arterial pressure reduced to 60 mm Hg) and then the rate of stimulation of the sciatic nerve was increased from 1 imp/sec to 4 imp/sec. An increase of the muscle exercise to this level was associated with a slight augmentation of blood flow and CFC increased to 0 039 (panel D) In addition, there was an increased net transfer of fluid into the muscle to judge from the inclining volume curve, but as before this phenomenon tended to decline gradually with time (observed in the interval between D and E) After raising transmural pressure 30 mm Hg (panel E) blood flow increased to 32 ml/min / 100 g reflecting in a more pronounced way than in C, the passive behaviour of the resistance vessels. CFC decreased to 0 030

Summarizing Fig 1 at rest, lowering transmural pressure resulted in relaxation of both resistance vessels and precapillary sphincters so that flow did not decrease despite the induced elastic recoil and CFC increased about 40 per cent During exercise with 1 twitch/sec at low transmural pressure, blood flow approximately doubled and CFC increased somewhat more than 35 per cent above the corresponding value at rest, indicating dilatation of resistance vessels and precapillary sphincters. Increasing transmural pressure during this level of exercise caused CFC to decrease by about 40 per cent, reflecting constriction of precapillary sphincters. In contrast, blood flow increased, indicating that the passive distention of the resistance vessels overcame any active response to increased transmural pressure. Increasing the metabolism by increasing the rate of stimulation to 4 imp/sec diminished, to a large extent, the effect of increased transmural pressure on precapillary sphincters. In this situation the pressure rise decreased CFC by only some 20 per cent. The passive behaviour of the resistance vessels was even more pronounced than at 1 imp/sec.

It is important to note that in the 5 expts. in which isometric tension was recorded, no decrease in contractile force was observed upon transition from low to high transmural pressure. This indicates that the constriction of the precapillary sphincters is not related to decreased metabolic rate

Fig 2 shows data from an experiment performed in order to evaluate more precisely the balance between passive and active changes of resistance vessels during alterations in transmural pressure at different metabolic levels. With the perfusion pressure kept constant at 50 mm Hg, transmural pressure was raised in 10 mm Hg steps first during rest, and then during work at 1 twitch/sec and 4 twitches/sec. These results are expressed as a plot of per cent change in flow against mean transmural pressure, i.e. $(P + P)/2$, where P = arterial inflow pressure and P = venous outflow pressure (Folkow and Löfving 1956)

The lowest dashed line connects points obtained with the muscle at rest While the first 10 mm Hg increase in transmural pressure caused a 4 per cent increase in flow subsequent increases in transmural pressure caused instead a decrease in flow These rather small changes indicate that the passive distending force is balanced nicely by the increased myogenic tone.

The upper two dashed lines show data obtained during exercise at 1 twitch/sec and 4 twitches/sec. The flow at 1 twitch/sec increased only slightly when mean trans-

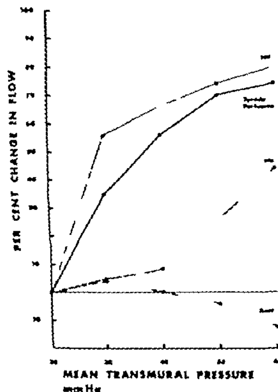


Fig. 2

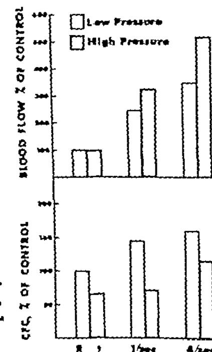


Fig. 3.

Fig. 2. The changes in flow associated with increases in mean transmural pressure (constant perfusion pressure) the muscle region rest, working 1 twitch/sec and 4 twitches/sec, and during perfusion with Tyrode's solution.

Fig. 3. Average values from 12 expts for CFC and flow low and high transmural pressure (difference averaged 28 mm Hg) during rest, and work 1 imp/sec and 4 imp/sec

mural pressure was raised to 35 and 45 mm Hg and the subsequent increases to 55 and 65 mm Hg resulted in 26 and 44 per cent increases in flow respectively. At 4 twitches/sec the first 10 mm Hg rise in transmural pressure caused an increase of 56 per cent in flow while subsequent rises resulted in flow increases of 66, 74 and 80 per cent.

The solid line is replotted from Folkow and Löfving experiments (1956) in which a muscle and skin preparation was perfused with Tyrode solution, which probably rendered the vascular bed completely passive. The fairly close correlation between this curve and the curve for 4 twitches/sec suggests that during great metabolic demand the resistance vessels are completely passive with respect to changes in transmural pressure. On the other hand, in the presence of lesser degrees of metabolic demand there appears to be a competition between the metabolic vasodilator influence and the myogenic constrictor response to increased transmural pressure.

Fig 3 is a histogram summarizing the data on flow (upper section) and CFC (lower section) from all experiments. All data are calculated in terms of per cent of the values obtained for resting muscle at low transmural pressure, designated the control period The height of each bar indicates the mean value for a given experimental manoeuvre Each experiment showed the same trend as that indicated by the bars (with the exception of flow at high transmural pressure during rest)

In the individual experiments the levels of low and high transmural pressures varied slightly but the induced change in transmural pressure was of roughly the same magnitude averaging 28 mm Hg The average value for blood flow in the denervated muscle region at rest was 9 ml/min $\times$ 100 g at a perfusion pressure of 100 mm Hg With regard to the resistance vessels, the outcome of the conflict between the distending force of increased transmural pressure and the myogenic response at rest was not predictable but averaged no change in blood flow or vascular resistance Elevation of transmural pressure caused an average decrease in CFC of 33 per cent from the contol value of 0 019

At low transmural pressure work at 1 twitch/sec always increased flow the average being a 2.4-fold increase Elevating transmural pressure caused a further increase in flow in all experiments, the flow then averaging 3.3 times the control value CFC also increased with exercise in all experiments, averaging 145 per cent of the control value. Elevation of transmural pressure, however reduced the CFC to 72 per cent of the control level, a value only slightly higher than CFC during high transmural pressure at rest Thus, in all preparations the myogenic response of the precapillary sphincters remained intact at this work level.

Increasing the rate to 4 twitches/sec caused an increase in flow in each case. At low transmural presure flow averaged 3.5 times control flow and at high transmural pressure, 5 1 times control flow CFC was, at low pressure, 160 per cent and, at high pressure, 120 per cent of the control value Thus flow was again increased when transmural pressure was elevated. CFC, on the other hand was still decreased upon transition from low to high transmural pressure, although it was decreased by only 24 per cent as opposed to 50 per cent during 1 twitch/sec.

As stated above, 2 experiments showed no change of precapillary sphincter tone when transmural pressure was altered In both of these experiments, the body temperature of the cat was below normal, although, in other respects, these experiments were technically sound Therefore 2 additional experiments were performed to test the possibility that lowered temperature could account for the decreased responsiveness. In one experiment, after demonstrating that increased transmural pressure caused a decrease in CFC, the temperature of the perfusing blood was allowed to drop from 37° to 33° C. This manoeuvre abolished the precapillary sphincter response. This finding was confirmed in the other experiment in which precapillary sphincter responsiveness to elevated transmural pressure was absent when first tested during perfusion with blood at 33 C, but returned when temperature was raised to 37° C.

Discussion

The results of the present study are in agreement with the concept that the vascular smooth muscle of the various functionally differentiated sections of the peripheral vascular bed has different characteristics (e.g. Folkow and Mellander 1964, Mellander 1964). Adopting Bozler's terminology (1948) it appears that multi-unit smooth muscle is present mainly in large precapillary resistance vessels and in capacitance vessels, while the single unit type is more prevalent in smaller precapillary resistance vessels and precapillary sphincters. This is in agreement with data suggesting that the control of the capacitance segment is largely neurogenic, that the resistance vessels are influenced by both extrinsic and local factors, and that the precapillary sphincters are chiefly controlled by local events (e.g. Folkow and Mellander 1964, Mellander 1964).

The present study indicates that of the two main local control mechanisms, metabolic demand is comparatively more important than transmural pressure in determining the tone of the resistance vessels, while the reverse is true for precapillary sphincters (Fig. 3). The influence of these two local factors is competitive and differences in their effects on resistance vessels and precapillary sphincters are largely quantitative. This finding is in agreement with general concepts of the mechanism of variability of smooth muscle response (Bohr 1965). It appears that the muscle of the smaller precapillary vessels rather closely mimics the behaviour of "primitive" non-innervated vascular smooth muscle such as that of the umbilical artery (Sparks 1964). Though the tone of the precapillary sphincters might be largely dependent upon automaticity in their single smooth muscle cells, the tone of the precapillary resistance vessels might, in addition, be dependent upon spread of excitation from pacemaker regions (Johansson and Bohr 1966). It is possible that metabolites can interfere with this propagation of impulses which could help to explain why the metabolic factor overrides the myogenic response in the resistance vessels.

Two explanations for the autoregulation of skeletal muscle blood flow have received wide support: these can be referred to as the metabolic hypothesis and the myogenic hypothesis. Previous studies have suggested that the metobolic factor dominates in autoregulation of blood flow and the present study clearly demonstrates that the metabolic factor can override the myogenic one. We would like to propose an extension of the concept of autoregulation to include autoregulation of capillary fluid filtration. This proposal involves two distinct local adjustments: first, regulation of the size of the capillary surface area available for exchange by means of changes in precapillary sphincter tone, and second, the regulation of mean hydrostatic capillary pressure via alteration of the precapillary/postcapillary resistance ratio. Autoregulation of filtration exchange may be accomplished by either of these mechanisms, depending on the tissue involved and the prevailing circumstances. For example when the transmural vascular pressure of skeletal muscle is altered at rest (Mellander, Öberg and Odelram 1964) or during exercise (Fig. 1 and 3) precapillary sphincter tone varies, and thus controls capillary surface area and filtration exchange. In the intestine on the other hand, autoregulation of filtration

exchange can be accomplished by shifts in precapillary/postcapillary resistance ratio so that mean capillary pressure is kept constant, as, for example, during vasoconstrictor fibre discharge (Folkow *et al.* 1963 Öberg 1964) This does not occur in skeletal muscle (Mellander 1960) The importance of the myogenic response of precapillary sphincters to increased intravascular pressure is obvious in the case of the circulation of skeletal muscle in which, during exercise, metabolic demand dominates the resistance vessels which, in turn, leads to a decrease of the precapillary/post capillary resistance ratio (Kjellmer 1964a) This latter effect *per se* would favour a rise in capillary hydrostatic pressure. In the absence of a myogenic response of the precapillary sphincters elevation of hydrostatic pressure during exercise could result in gross edema. This consideration may be especially important for the circulation of the legs of man during exercise in upright posture.

An increase in precapillary sphincter tone at high transmural pressure during exercise would appear to result in some decrease in the efficiency of the nutritional circulation through skeletal muscle Capillary surface area as measured by CFC, however is not a direct indication of diffusion distances, which may be affected much less than capillary surface area. Precapillary sphincters may be opening and closing asynchronously so that capillary surface area is decreased but flow distribution is still fairly uniform throughout the whole muscle over a reasonable length of time. In addition the filtration which does occur during exercise at high transmural pressure may be important in improving capillary nutritional exchange, since Mellander and Lundgren (1966) have demonstrated that tissue-blood transport can be significantly increased in the presence of net filtration.

During moderate exercise at elevated transmural pressure blood flow increased over threefold, while there was virtually no increase in CFC above rest level (Fig 3) Such an increase in blood flow over a restricted capillary section would require either a decreased capillary resistance to flow or an increased pressure gradient over the capillary section. The increased flow might be diverted through non-nutritional channels, but there is evidence which denies their existence in skeletal muscle e.g Dieter 1954 Piper and Rosell 1961) The diameters of the patent capillaries might increase as a result of the raised transmural pressure. Although modern anatomi investigations of the microcirculation deny the occurrence of significant changes in capillary bore to alterations in transmural pressure (Zweifach, personal communication) even very small increases might decrease capillary resistance considerably if the Poiseuille relationship applies to this vascular section An increase in blood flow velocity can be associated with a decrease in anomaleous viscosity of blood, but this phenomenon is only seen at very low flow velocities further this hardly applies to capillaries where a "bolus" flow is usually existing A factor of greater importance is probably the change in pressure drop across the capillary bed that occurs during exercise. At rest, this pressure drop is quite small, perhaps even smaller than generally assumed. During exercise, however there is a pronounced relaxation of precapillary resistance vessels which must lead to quite a significant rise in pressure on the arterial side of the capillary It is conceivable that a threefold increase in

blood flow through a fairly restricted capillary bed can be accounted for by this increased capillary pressure drop and by a somewhat decreased resistance in patent capillaries.

When the vascular bed was exposed to the given increase in transmural pressure CFC decreased by an average of 33 per cent in the resting muscle but by an average of 50 per cent at exercise at 1 twitch/sec (Fig 3) The myogenic response of the precapillary sphincters thus appears to be greater at this work level, despite the concomitant competitive effect of vasodilator metabolites. This finding might suggest that optimum myogenic responses are elicited above a critical pressure level Exercise *per se* decreases the precapillary/postcapillary resistance ratio so that, at constant arterial inflow pressure and venous outflow pressure mean hydrostatic capillary pressure can rise by as much as 10 to 15 mm Hg (Kjellmer 1964a *cf* also Fig 1) The prevailing pressure at the precapillary sphincter level is thus probably somewhat greater in exercise than at rest in the present experiments. Therefore a superimposed given increase in transmural pressure might be a more effective stimulus for precapillary sphincter constriction in exercise than at rest. The pressure/flow curve at rest in Fig 2 might indicate similar characteristics for the myogenic response in the resistance vessels shifting transmural pressure from 25 to 35 mm Hg produced slight augmentation in flow while changes from 45 to 55 and from 55 to 65 mm Hg elicited decreases in flow The above hypothesis deserves further investigation.

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Isolation of a Mast Cell Degranulating Polypeptide from Ascaris suis

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Abstract

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Ascaris suis was extracted with 60% ethanol in dilute formic acid after previous removal of fat with organic solvents. After dialysis, the extract was further purified by ammonium sulphate precipitation, treatment with Amberlite IRC-50 (XE-64) chromatography on CM cellulose and gel filtration on Sephadex G-25 The purified product with molecular weight probably within the range 2000—3000 had basic character with an isoelectric point at 12.3 in 0.05 M borate buffer. I behaved as nearly homogeneous compound on paper electrophoresis and on gel filtration. Nitrogen content 16.7 %. Fifteen amino acids were identified by paper chromatography after acid hydrolysis alanine, arginine, cystine, glycine, histidine isoleucine, leucine lysine methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. The purified product was degraded and inactivated by the proteolytic enzymes papain, pronase, trypsin, α-chymotrypsin and partially by pepsin. The polypeptide caused degranulation of rat mesentery mast cells, even such cells from germ free animals, indicating that the mast cell response was he result of direct action of the *Ascaris* principle on the mast cell and not due to an antigen antibody reaction.

It has been well established that polymer amines such as compound 48/80[1] and antigens (horse serum, serum albumin etc.) cause degranulation of rat mast cells with the subsequent release of histamine A "histamine liberator" degranulating mast cells by an identical or a very similar mechanism as the agents mentioned was found to be present in extracts of *Ascaris* (Uvnäs *et al.* 1960) Various authors have reported on the toxic effects of *Ascaris* extract, and they all agree that the effect caused by the action of such extracts closely resembl an anaphylactic shock reaction (Shimamura and Fuji 1917 Macheboeuf and Mandoul 1939 Rocha e Silva and Graña 1946 Beraldo *et al* 1961) Since it i known that *Ascaris* harbours substances with antigenic properties, the toxicit has been a cribed t these compounds. This report describes the preparation purific tio n and some of the chemical properties of a presumably non-antigenic mast cell degranulating principle from *Ascaris suis*

[1] The product obtained by the condensation of p-methoxy N-methyl phenethylamine with formaldehyde (Baltzly *et al* 1949

Material and methods

Swine Ascaris was obtained from "Slakteriföreningen" Uppsala. The worms were frozen immediately after collection and were kept at — 20° until extraction.

Preparation of Ascaris L. tract. Ascari worms (500 g) were cleaned in tap water, cut into small pieces, transferred to a Büchner funnel and the body liquid removed by suction. The material, suspended in acetone, was further disintegrated in a homogenizer and then pre-extracted successively with acetone, chloroform — methanol (4 : 1 v/v) and ether respectively, 15 min. (3 l) of each solvent at 30—40° under vigorous stirring. The organic extracts were discarded. The residual de-fatted material (ca 350 g) was stirred for 1 hr with 60 % ethanol in 0.2 N formic acid at 60—65°. The extract was sieved through muslin, cooled and centrifuged. A further two consecutive extractions were performed in this way, 500—600 ml solvent being used each time. The combined supernatants were concentrated *in vacuo* to 100 ml. To this solution was added ethanol (15 volumes) with rapid stirring. After 1 hr at 0 —5° the precipitate was removed by centrifugation and the supernatant was again concentrated *in vacuo* until complete removal of the alcohol was achieved. The turbid liquid was shaken 3 times with excess ether, the ether phases being discarded. The precipitate formed was centrifuged, and the clear yellow supernatant dialyzed against running tap water over night and then against 0.005 N HCl for 4—6 hrs. Concentration and lyophilization of the dialyzed solution furnished a crude extract (= Product A) 0.3—0.4 % of the starting material (the de-fatted material).

Purification procedure. To the dialyzed product (ca 1 g) in aqueous solution (100 ml) was added with stirring solid ammonium sulphate (47 g) giving 70 % saturation. After 4 hrs at 0 —5° the mixture was centrifuged. The supernatant was discarded, the precipitate dissolved in water and dialyzed overnight against running tap water. The solution was acidified (hydrochloric acid) to pH 2—3, heated in a boiling water-bath for 15 min, cooled, then neutralized (sodium hydroxide) to pH 8—9 and quickly poured into a suspension of Amberlite IRC-50 (XE-64) H+ (3 g) in water. The mixture was diluted with water to 2 l and stirred for 2 hrs; the resin was filtered off, washed exhaustively with water, transferred to a chromatography column and eluted with 2 N formic acid (50 ml). The acid eluate was concentrated *in vacuo* and the formic acid removed by repeated distillation with water. To the resulting solution (ca 10 ml) was added acetone (8 vol) with stirring. After 2 hrs at 0 —5° the precipitate was centrifuged off and dissolved in 0.075 M ammonium bicarbonate. The sample was applied to a CM-cellulose column in the ammonium form and eluted stepwise with aqueous ammonium bicarbonate, pH 7.9 (Stott 1959). From each fraction 0.25 ml was withdrawn for biological testing and 0.1 ml for the Folin-Lowry test (Lowry *et al.* 1951). The active fractions were pooled, concentrated and the buffer removed by lyophilization. The product, dissolved in dilute formic acid, was percolated through a Sephadex G-25 column with 0.02 N formic acid, as the eluant, the fractions being subjected to the same tests as the eluates from the CM-cellulose column. Finally the active fractions were pooled and the product B isolated by freeze-drying.

The test for mast cell degranulating activity was carried out by the rat mesentery test as described by Högberg and Uvnäs (1960) with the exception that the incubation pH was changed from 7.4 to 6.8.

Enzymes. Trypsin, 2 × cryst., (Mann Research Laboratories) Pepsin 2 × cryst., (Mann Research Laboratories) — chymotrypsin, B-grade (California Corp. for Biochem. Research) Pronase, B-grade, (California Corp. for Biochem. Research.) Papain 2 x cryst. (Worthington Biochem. Corp.) *Polymyxin B* (Novo, Copenhagen)

Enzyme experiments were carried out under toluene in the appropriate buffer system at 38° for 18 hrs. The digest was finally acidified, heated for 5 min in a boiling water bath, cooled, and centrifuged if necessary. The resulting solution was subjected to the rat mesentery test, and to paper chromatography.

The CM-cellulose column (Whatman powder CM 70) was prepared according to Colowick and Kaplan (1962).

Acid hydrolysis was effected by heating the sample (mg) in 6 N HCl (0.5 ml) at 105° for 20 hr in a sealed tube.

Paper chromatography was carried out on Whatman no. 1 filter paper with the solvent systems A: n-butanol : acetic acid : water (60 : 15 : 25 v/v) or B: sec-butanol : 3 % ammonium hydroxide (3 : 1 v/v). For two-dimensional separations the chromatogram was first developed with solvent A, then with phenol : water (100 : 25 w/v). Ninhydrin was used routinely as location reagent.

Paper electrophoresis was performed on a LKB 3 76 apparatus employing Schleicher and Schüll No. 2043 filter paper, a running time of 6 hr and a current of 0.3 m A/cm. Runs were carried out in 0.05 M phosphate or in 0.05 M borate buffers, in the pH range 6—12.4. Amido black or ninhydrin was used as location reagent.

Results

The dialyzed crude extract, product A, consisted of a heterogeneous material that could be separated into a number of components by paper electrophoresis. Paper chromatographic examination of an acid hydrolysate of the product revealed a large number of amino acids. Careful acid hydrolysis (1 N hydrochloric acid at 100° for 4 hrs) followed by paper chromatography showed the presence of glucose (aniline oxalate spray) probably arising from residual glycogen.

Apart from this carbohydrate contaminant no constituents other than amino acids could be dectected after acid hydrolysis, indicating that the product consisted almost entirely of protein/polypeptide material.

An initial fractionation was effected with ammonium sulphate, the precipitate containing the mast cell degranulating factor. This was firmly bound to the weak cation exchange resin Amberlite IRC-50 (XE-64) at neutral pH-values and could be eluted with acid or alkali while the bulk of impurities was washed out with water. Because of the instability of the biological activity in an alkaline medium acid was employed for elution. Chromatography on calcium phosphate (hydroxyl apatite) and on DEAE-cellulose was tried to obtain a more extensive fractionation of the *Ascaris* extract, but without success. However further purification was achieved by ion exchange chromatography on CM-cellulose in the salt form. Impurities were eluted with dilute ammonium bicarbonate while the mast cell degranulating compound required stronger electrolyte concentration to release it from the column. Some residual inactive material was eluted with 0.5 M buffer (Fig. 1).

The final step of the purification procedure, gel filtration through a Sephadex G-25 column, led to a further slight fractionation of the organic material apart from removing inorganic salts present. Essentially the curve of the mast cell degranulating activity coincides with the Folin-Lowry curve (Fig. 2). Incorporation of 8 M urea in the elutant did not cause any alteration of the elution pattern.

On paper electrophoresis product B exhibited a distinct basic character moving as a single compound towards the cathode in 0.05 M phosphate buffers of pH between 6 and 10. When run in 0.05 M borate buffers at higher pH-values it travelled as a cation up to pH ≈ 12.5 where the material remained at the starting line. Some

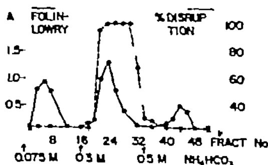


Fig. 1. CM-cellulose column (1.2 × 25 cm). The sample (ca. 100 mg of the acetone precipitate dissolved in 0.075 M ammonium bicarbonate (2 ml). Stepwise elution with ammonium bicarbonate 0.075 M, 0.5 M and 0.5 M, 75 ml of each solution. 5 ml fractions collected. Fractions 22—31 pooled and isolated.

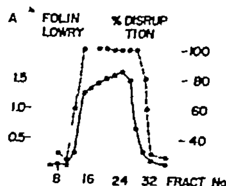


Fig. 2. Sephadex G-25 column (1.7 × 50 cm). The product from the CM-cellulose column (6 mg) dissolved in 0.02 N formic acid (1 ml). Elution with 0.02 N formic acid. 3 ml fractions collected. Fraction 13—29 pooled and isolated.

tailing both in the anodic and in the cathodic direction was observed indicating only a slight degree of heterogeneity. When comparing the *Ascaris* product B with other potent mast cell degranulating compounds such as phospholipase A and polymyxin B they all showed an almost uniform electrophoretic mobility demonstrating that the three substances have an isoelectric point in the pH range 12.1—12.4 in the 0.05 M borate buffer system. Compound 48/80 although possessing no isoelectric point, also travelled on electrophoresis very similarly to the compounds mentioned. On chromatography on Amberlite IRC-50 (XE-64) as well as on gel filtration through a Sephadex G-25 column the polypeptide antibiotic polymyxin B closely resembled the *Ascaris* principle in its behaviour.

Product B contained nitrogen 16.7 % (micro-Kjeldahl), phosphorus 0.09 % (Bartlett 1959) and gave a nearly negative test for sulphydryl groups, (SH-content of the order 10^{-4} mmoles SH/g) (Jocelyn 1962). Neither product B nor its hydrolysis products showed any fluorescence under the U.V. lamp, and no reaction was obtained with the Dragendorff reagent, indicating the absence of quaternary ammonium compounds.

Acid or alkaline hydrolysis of the purified *Ascaris* extract followed by exhaustive extraction of the hydrolysate with ether and subsequent paper chromatography of the evaporated extracts (solvent B) did not reveal any spots of lipid nature (Rhodamine 6 G spray). The U.V. absorption spectrum of product B resembled that of a tyrosine-containing polypeptide. In acid solution (0.1 N hydrochloric acid) it exhibited a maximum at 275 mμ with the characteristic shift to longer wavelenght, 290 mμ at alkaline pH (0.1 N sodium hydroxide). In alkaline solution the distinct tyrosine-absorption peak at 242 mμ was also clearly observed.

Paper chromatography of an acid hydrolysate of the product B showed the presence of the following amino acids: alanine, arginine, cystine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. Judging from the intensity of the spots the contents of alanine, arginine, glycine, leucine were large compared with the other constituent acids.

Product B was soluble in water and in water-ethanol mixtures containing up to 80 % of alcohol. It was insoluble in the common organic solvents such as chloroform,

TABLE I.

Product	µg/ml	% Degranulation
A	20	60— 70
B	1	80—100
Polymyxin B	0.4	90—100

TABLE II.

Substrate	Trypsin	α-Chymotrypsin	Pepsin	Papain	Pronase
Prod. A	—	—	—	+	+
Albumin	+	+	+	+	+
Prod. A + Albumin	—	—	—	+	+
Prod. B	+	+	(+)	+	+
Polymyxin B	—	—	—	(+)	(+)

Substrate (5 mg) and enzyme (1 mg) employed.
— no proteolysis, + proteolysis, + proteolysis and loss of activity (+) partial proteolysis and partial loss of activity

ether acetone, ethylacetate and n-butanol. It could be heated at 100 under reflux in 0.03 N hydrochloride acid for 1 hr without loss of activity After 2—3 hrs the compound was gradually destroyed, and some free amino acids and peptides were observed on paper chromatography

The mast cell degranulating activity of the *Ascaris* products A, B and of polymyxin B is seen in Table I

Purified *Ascaris* extract caused normal degranulation of mesentery mast cells from germ-free rats, i.e. rats free of antibodies thus demonstrating the non antigenic nature of the active principle (Backlund, Gustafsson and Uvnäs, to be published)

The crude *Ascaris* product (A) resisted the action of the common proteolytic enzymes trypsin, α-chymotrypsin and pepsin Not only did the mast cell degranulating activity remain intact but the whole of the material proved resistant to proteolysis Even after addition of bovine serum albumin to the substrate the enzymes exerted no proteolytic effect On the other hand incubation with papain or with pronase (Nomoto *et al* 1960) caused considerable hydrolysis of A with a concomitant decrease of activity The purified product B was rapidly degraded and inactivated by papain and pronase and also by trypsin α-chymotrypsin and to a small extent by pepsin. Polymyxin B was partl hydrolyzed by papain and pronase, but was not affected by the other th ee enzymes Table II)

Discussion

The present results clearly show the polypeptide character of the *Ascaris* mast cell degranulating principle. No constituents other than amino acids could be detected and the general properties correspond well to those of an amino acid polymer.

The resistance to fractionation usually constitutes a major obstacle in the examination of polymer molecules, and in the present studies the main problem has been that of obtaining the active principle in a pure state. A considerable purification has been achieved, the purified product behaving essentially as a homogenous compound on paper electrophoresis, and the elution pattern from the Sephadex G-25 column showed only one Folin Lowry peak, although this was somewhat broad.

Hydrogen bonding frequently hampers the separation of proteins and other macromolecules by chromatography. However, the presence of urea in high concentrations, known to eliminate hydrogen bonds, resulted in no further fractionation. Although conclusive evidence is lacking for the homogeneity of product B it was considered pure enough for further examination.

The nitrogen content (16.7%) is a normal figure for an amino acid polymer whereas the finding of phosphorus (0.09 %) frequently present in varying amounts in protein material probably can be regarded as insignificant.

The practically negative test for free sulphydryl groups suggests that the cystine present in the acid hydrolysate occurs as such also in the polymer and is not formed by the oxidation of cysteine residues. Noteworthy is the fact that all fifteen amino acids identified were either neutral or basic, a finding which agrees with the high isoelectric point of the compound.

Concerning the molecular weight (M.W.) of product B the only information so far stems from the gel filtration experiments. The mast cell degranulating principle was considerably retarded on Sephadex G-75 and on G-50 and a marked retardation was observed also on G-25 indicating a M.W. of less than 5000. The danger must be emphasized, however, of drawing too far-reaching conclusions from these experiments. Under certain conditions adsorption to the gel matrix occurs, leading to abnormal elution rates and subsequent misinterpretation of the results. However, the purified *Ascaris* extract showed nearly identical elution rates at acid and neutral pH values and also at different salt concentrations suggesting that the elution pattern observed is the "true" one. On Sephadex G-25 the polypeptide antibiotic polymyxin B (M.W. 1202) was slightly more retarded than product B; thus for the latter a M.W. somewhere between 1200 and 5000 may be expected. Considering that fifteen amino acids occur in the molecule and that some of them constitute more than one residue it seems reasonable to assume a M.W. in the range of 2000—3000 for the *Ascaris* polypeptide. Rather surprisingly it was found that the *Ascaris* product A resisted the action of trypsin, α-chymotrypsin and pepsin, and furthermore that the product completely inhibited the proteolysis of albumin by these enzymes, (Table II).

Ascaris in its natural environment is subject to attack by various proteolytic enzymes against which it possesses effective defence. The parasite produces potent inhibitors of pepsin, trypsin and chymotrypsin and these inhibitors are reported to be

of protein/polypeptide nature (Collier 1941 Green 1957 Peanasky and Laskowski 1960, Peanasky and Szucs 1964)

After purification, however the *Ascaris* product could no longer resist the action of trypsin, α-chymotrypsin and pepsin There was some difference in the degree of proteolysis caused by each of the three enzymes. Digestion with trypsin resulted in complete degradation, α-chymotrypsin proved somewhat less effective while pepsin had only a slight effect. It is thought that product B with its high contents of arginin and lysine makes a good substrate for trypsin whose specificity in volves arginine or lysine residues. On the other hand pepsin requires tyrosine or phenylalanine for its hydrolytic activity and judging from the paper chomatography these aromatic amino acids are present only in minute amounts.

For comparison, polymyxin B was incubated with the same series of enzymes. As seen from Table II this substance proved rather inert and was not even severely affected by the powerful protease pronase no doubt due to the unique composition of this antibiotic with its high content of α,γ-diamino-n-butyric acid.

The reason for the present interest in polymyxin B is that it is the most potent mast cell degranulating polypeptide of known chemical structure (Vogler *et al.* 1964) This antibiotic has been reported to be an active mast cell degranulating agent both *in vitro* (Lagunoff and Benditt 1960) and *in vivo* (Bushby and Green 1955) It was of interest to see if the *Ascaris* polypeptide possessed any of the characteristic features of the antibiotic. However neither α,γ-diamino-n butyric acid nor the fatty acid 6-methyl octanoic acid or any other lipid constituent could be detected. Whether the *Ascaris* principle, like the polymyxins, has a cyclic structure can not be decided without further structural analysis.

A comparison of the two compounds would suggest that the electrical charge is the only point at which they show any great similarity Moreover their M.W s are roughly within the same range.

Newton (1955 1956) studied the action of the polymyxins in bacteria. He found that polymyxin-sensitive micro-organisms on contact with the antibiotic were degranulated apparently in a way similar to mast cell degranulation. In bacteria the action of polymyxin is believed to be due to its ability to combine with structures within the bacterial cell responsible for the maintenance of the osmotic equilibrium of the cell. Good evidence exists that the antibiot c affects the osmotic equilibrium by reacting with ionized phosphat groups of phospholipid components of the membrane underlying the bacterial cell wall. In view of this theory it seems that the basic character of the antibiotic is of great importance for its effect. At present nothing appears to be known about the mode of action of polymyxin on mast cells. However it should not be considered inconceivabl that this compound — and also other basic histamine liberators as the *Ascaris* polypeptid — act by combining with acid groups in the mast cell membrane changing its electrical charge ultimately leading to discharge of granules and release of histamine The mechanism by which this transient change in the permeabil ty of the cell membrane is produced is so far unknown. Energy is evidently required for the mast cell response since it is

reversibly blocked by anoxia and other types of metabolic inhibitors (Uvnäs et al. 1960)

The fact that the *Ascaris* principle causes degranulation of mast cells isolated from germ free rats is of great principal importance. Germ free rats have no antibody production and the response of mast cells from such rats cannot therefore be ascribed to an antigen — antibody reaction but must be due to a direct action of the *Ascaris* polypeptide on the mast cell surface. *Per analogiam* it might be assumed that other allergic" reactions to primitive animals such as caterpillars, parasites, shellfishes etc. and even to some foods might be due to their content of basic polypeptides producing mast cell degranulation by direct action, not requiring previous sensitisation.

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Quantitative in Vitro Studies on Noradrenaline Uptake and its Inhibition by Amphetamine, Desipramine and Chlorpromazine

By

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Abstract

HÄGGENDAL, J. and B. HAMBERGER. *Quantitative in vitro studies on noradrenaline uptake and its inhibition by amphetamine, desipramine and chlorpromazine.* Acta physiol. scand. 1967. *70.* 277—280.

The accumulation of noradrenaline (NA) was determined biochemically after incubation with NA of slices from the vas deferens, cerebral cortex and neostriatum of rats pretreated with reserpine and nialamide. It was found that both areas rich in NA terminals, such as the vas deferens and cerebral cortex, and areas rich in dopamine (DA) terminals, such as the neostriatum, efficiently accumulated NA. (+)-Amphetamine effectively inhibited NA accumulation in all the tissues, whereas desipramine inhibited the uptake in areas rich in NA terminals efficiently, but in areas rich in DA terminals only to a slight extent. In this respect, the effect of chlorpromazine was similar to that of desipramine. The data strongly support the view that the reserpine-resistant accumulation mechanism of the transmitter differs in the two types of catecholamine terminals.

Peripheral tissues are known to have the ability to accumulate injected catechol amines also after reserpine pretreatment, provided that monoamine oxidase (MAO) has been inhibited (see e.g. Malmfors 1965, Carlsson 1966). In the brain, similar studies cannot be made *in vivo*, due to the blood-brain barrier, except in certain regions (Fuxe and Hillarp 1964, Fuxe, Hamberger and Malmfors 1966). Studies *in vitro* on isolated brain tissues have provided strong histochemical evidence that the central catecholamine-containing neurons also have a reserpine-resistant uptake-concentration mechanism (Hamberger and Masuoka 1965, Hamberger 1967) located at the level of the cell membrane (Malmfors 1965). In the present study the uptake of noradrenaline (NA) in slices of brain and vas deferens after reserpine pretreatment was determined biochemically. The effects of (+)-amphetamine, desipramine and chlorpromazine on this uptake were also studied.

Material and methods

Male Sprague Dawley rats (180—200 g) were pretreated with reserpine (Serpasil® Ciba, 10 mg/kg, 12—18 hrs) and the MAO inhibitor nialamide (Niamid® Pfizer 100 mg/kg, 1 hr) The rats were killed by exsanguination under light ether anaesthesia. Slices of the cerebral cortex, neostriatum and vas deferens were incubated i 10 ml of a Krebs-Ringer bicarbonate buffer solution containing glucose (1.8 mg/ml) EDTA (ethylenediaminetetraacetic acid, disodium salt, 0 05 mg/ml) and ascorbic acid (0.2 mg/ml) and saturated with carbogen gas (93.5 % O_2, 6.5 % CO_2) as described earlier (Hamberger 1967) The tissue was first preincubated for 15 min, without or in the presence of (+)-amphetamine (sulphate, Sigma, 2 7 × 10^{-}[illegible] M) desipramine (Pertofran® Geigy 10^{-}[illegible] M) or chlorpromazine (Hibernal® Leo, 10^{-}[illegible] M) NA /(—)-NA hydrochloride, Sigma/ was added to give final concentration of 0.1 µg/ml, calculated as the base. Incubation was continued for 60 min. The tissue slices were then rapidly rinsed in fresh buffer followed by incubation in new buffer without NA for 10 min. The slices were then blotted on filter paper weighed, and homogenized in 5 ml of ice-cold perchloric acid. Control tissue which had not been incubated with NA was included in the experiment. Slices from one or two rats were pooled for each sample. Samples were also taken from th media at the end of incubation (60 min after the addition of NA) The NA content was determined fluorometrically according to Häggendal (1963)

Results

After incubation with NA, the vas deferens, cerebral cortex and neostriatum had a considerably higher NA content than the controls (Table I) The largest amount was present in the neostriatum. After (+)-amphetamine, all three tissues had a low NA content, whereas incubation with desipramine and chlorpromazine resulted in a low concentration in the vas deferens and cerebral cortex, but a high amount in the neostriatum.

The NA content of 7 media was also determined 60 min after addition of NA to the bath, with or without the drugs studied The lowest value 0 06 µg/ml was noted in a medium where slices of neostriatum had been incubated with NA only and a considerable amount was present in the slices (see Table I) The sum of the amount of NA in both medium and slices was found to be 0 094 ± 0 0076 µg/ml (mean ± ± s.e.m.) No value was below 0 078 µg/ml No corrections were made for losses of NA during the estimation procedures.

TABLE I Amounts of noradrenaline (µg/g) in tissues incubated *in vitro* with noradrenaline (NA) with or without addition of (+)-amphetamine, desipramine and chlorpromazine The tissues were taken from animals pretreated with reserpine and nialamide.

Treatment	Tissue		
	Vas deferens	Cerebral cortex	Neostriatum
Without NA	0.18 ± 0 031 (6)	0.04 ± 0.018 (5)	0 05 ± 0 010 (4)
NA	1 17 ± 0.297 (5)	0 18 ± 0 045 (5)	3 68 ± 0 689 (4)
(+)-Amphetamine $2.7 \times 10^{-}$[illegible] M + NA	0.11 ± 0.014 (5)	0.07 ± 0.015 (5)	0.18 ± 0 056 (4)
Desipramine 10^{-}[illegible] M + NA	0.11 ± 0.052 (4)	0.06 ± 0.020 (4)	1.81 ± 0 669 (4)
Chlorpromazine 10^{-}[illegible] M + NA	0 24 ± 0.105 (5)	0 05 ± 0.010 (5)	2 37 ± 0 560 (2)

The values are given as mean ± s.e.m. Number of experiments within brackets

Discussion

In the tissues studied, there is a network of catecholamine terminals. In the vas deferens and cerebral cortex they consist of NA terminals whereas the neostriatum contains mainly DA terminals (for references, see Hamberger 1967). It has been shown histochemically that, under the present conditions, a reserpine resistant uptake occurs in those nerve terminals which normally contain catecholamines. The main part of the noradrenaline found in this study after incubation with NA can therefore be considered to be localized within the nerve terminals. However it has been established histochemically that cells in the endothelial wall can accumulate catecholamines *in vitro* although at higher concentrations in the medium than those used here (Hamberger 1967).

The uptake of NA in brain slices from normal animals has been studied with labelled amines (Dengler *et al.* 1962, Ross and Renyi 1964). It was presumed that simple diffusion occurs when incubating tissue in concentrations from 0.1—0.2 µg/ml, whereas there is an active accumulation at lower concentrations (Dengler *et al.* 1962). However in the present study a distinct difference was present between the NA uptake without and with e.g. (+)-amphetamine at a NA concentration of 0.1 µg/ml. When studying the accumulation of NA at high concentrations in the bath, washing out of the passively diffused NA seems to be of importance for disclosing the specific uptake.

The recovery of NA in the present experiments appeared to be high, since about 90 % of the amount added was found in the medium and the slices. This figure is interesting since, in other *in vitro* experiments, only about 70 % of the isotope in the slice and less than 35 % of that in the medium was unchanged NA (Dengler *et al.* 1962). In the present study the inhibition of monoamine oxidase may partly explain these differences. A spontaneous destruction of catecholamines may also occur in experiments of this type (Häggendal and Svedmyr 1967). In our experiments, EDTA and ascorbic acid were added in order to protect the NA.

The present experiments showed that (+)-amphetamine prevented the *in vitro* accumulation of NA efficiently in both NA and DA terminals. The effect is probably due to both an inhibition of uptake and a release of accumulated NA (Carlsson and Waldeck 1966). In the concentrations used, desipramine and chlorpromazine appeared to inhibit the uptake in NA terminals, whereas the effect on DA terminals — if any — was of a much lower degree. Parallel histochemical experiments have produced the same type of inhibition pattern (Hamberger 1967). That the NA levels in the neostriatum were in fact, lower in the presence of desipramine and chlorpromazine than the control values may have been due to the presence of a certain amount of NA terminals in the material from the neostriatum. It may also well be that the extraneuronal binding had been inhibited (cf. Hamberger 1967). Histochemically it has been shown that higher desipramine concentrations (10^{-4}M) did not inhibit the uptake in DA terminals, while an increase in chlorpromazine concentration markedly inhibited this uptake (Hamberger 1967).

(+) Amphetamine, desipramine and chlorpromazine are known to prevent the accumulation of NA in both central and peripheral NA terminals (see e.g. Dengler Spiegel and Titus 1961 Iversen1965, Malmfors 1965 Carlsson 1966, Carlsson *et al.* 1966) Blockade of the uptake appears to be one of the basic explanations of e.g. the high NA levels in blood plasma of patients during physical activity and long-term treatment with high doses of chlorpromazine and imipramine (Carlsson *et al.* 1967 and unpublished data)

The present experiments focus further attention on the circumstance that certain drugs, above all the antidepressive imipramine-like drugs, do not inhibit the uptake of NA into DA terminals (cf. Carlsson *et al.* 1966, Fuxe, Hamberger and Malmfors 1966 Hamberger 1967) This implies that the reserpine-resistant accumulation mechanisms in NA and DA terminals are different.

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Acta physiol. scand. 1967. 70. 281—289

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Effect of Dihydralazine on Plasma Renin Activity and Renal Function during Supine Exercise in Normal Subjects

By

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Abstract

BOŽOVIĆ, L. and J. CASTENFORS. *Effect of dihydralazine on plasma renin activity and renal function during supine exercise in normal subjects.* Acta physiol. scand. 1967. *70.* 281—289

Plasma renin activity, clearance of inulin (C_{IN}) and para-aminohippuric acid (C_{PAH}), urinary sodium and potassium excretion during supine exercise were studied in normal healthy men. 8 subjects were treated during exercise with dihydralazine (Nepresolin® i.v.) and 8 subjects were untreated (control group). In the untreated subjects there was a statistically significant decrease in C_{PAH} and increase in filtration fraction. The decrease in C_{PAH} during exercise was less in the dihydralazine-treated group and the increase in filtration fraction almost inhibited. Urinary sodium excretion decreased significantly in both groups during exercise but returned slowly to pre-exercise level in the dihydralazine group. Plasma renin activity increased during exercise in both groups, most markedly in the dihydralazine group. After exercise plasma renin activity decreased to pre-exercise level in the control group but continued to increase in the dihydralazine group in spite of the increased renal blood flow. The findings indicate more marked sympathetic activity in the dihydralazine group and it is suggested that the sympathetic nervous system may have a direct influence on renin release irrespective of its renal hemodynamic action.

Muscular exercise increases plasma renin activity (Helmer 1964, Božović, Castenfors and Piscator (1967). The mechanism responsible for the release of renin from the kidney remains obscure, but some theories (Kohlstaedt and Page 1940, Tobian 1964, Skinner, McCubbin and Page 1964) suggest that the renal vasoconstriction occurring during exercise may be responsible for the renin release.

To test this possibility the present investigation was undertaken to study the effect of a renal vasodilatory drug on plasma renin activity during exercise. Dihydralazine (Nepresolin® CIBA) was chosen because of its pronounced renal vasodilatory effect (Reubi 1950) and its inhibitory effect on renal vasoconstriction during exercise (Werkö *et al.* (1954). Renal hemodynamics, water diuresis and urinary sodium and potassium excretion were also measured.

TABLE I Age, height, weight, W_{170} of all subjects

Subject	Age years	Height cm	Weight kg	W_{170} kpm/min
1 SV	27	170	69	1 100
2 SV	23	170	71	1,000
3 PB	27	182	73	850
4 DW	21	181	78	1 100
5 HW	25	185	68	1 100
6 CS	22	186	75	800
7 UL	24	182	74	1,200
8 BW	22	174	58	1 000
9 EG	24	184	84	1 000
10 BI	22	181	65	850

TABLE II Work load, hydration and heart rate recorded at the end of the 45-minute exercise

Dihydralazine group					Control group				
Subj.	Load Kpm/min	% W_{170}	Heart rate	Hydration ml	Subj	Load kpm/min	% W_{170}	Heart rate	Hydration ml
VS	800	73	162	2,480	VS	900	82	163	2,400
SV	700	70	170	2,290	SV	900	82	172	3 020
PB	500	59	168	2,930	PB	600	71	146	2,260
DW	650	59	180	3,040	DW	700	64	159	4,220
HW	800	73	176	2,750	HW	800	73	158	2,742
CS	500	62	158	1,800	CS	600	75	152	2,680
UL	600	50	170	2,725	EG	800	80	162	2,700
BW	600	60	165	2,100	BI	700	82	167	2,750
Mean	644	63	169	2,500	Mean	750	76	160	2,830

Material

10 healthy male volunteers, age 20—27 years, were investigated. All were well informed about the procedure of the experiment. Their work capacity was determined prior to the test as the amount of work they could perform on a bicycle ergometer at a heart rate of 170 beats/min (W_{170}) (Sjöstrand 1947). Table I shows the age, height, weight and W_{170} of the subjects in the study. Six subjects underwent 2 tests each, one with and one without dihydralazine. Four subjects underwent only one test, two with dihydralazine and two without.

Procedure and methods

Exercise was performed in the supine position for 45 min. C_{PAH}, C_{IN}, urine flow, C_{osm}, C_{H_2O}, $\frac{U}{P}$, *urinary sodium and potassium excretion were measured before*, during and after the exercise according to the methods and procedure described elsewhere (Castenfors 1967). *Plasma renin activity* was determined by the method of Boucher *et al.* (1964) with the modification that heparin was used as anticoagulant instead of EDTA.

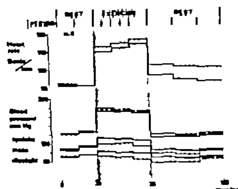


Fig. 1



Fig. 2.

Fig. 1 Effect of dihydralazine on heart rate and blood pressure during and after exercise. Mean values of 8 subjects are given dihydralazine group (– – –) control group (———).

Fig. 2. Effect of dihydralazine on C_{PAH}, C_{TF} and FF during and after exercise. Per cent change and significant difference of all mean values compared to period 2 are given dihydralazine group (– – –) control group (———).

I 8 expts. 12.5 mg Nepresolin® was given l the start and in the middle of the 45-min exercise period. 8 expts. (control group) were performed without drug treatment. The individual work load was selected to produce heart rate at the end of exercise of approximately 160 beats/min. I order to fulfil this criterion lower work load had to be chosen in the Nepresolin-treated group than in the control group. (Table II shows the individual work load, hydration and heart rate at the end of the 45-min exercis period in all experiments.)

I 3 subjects in the control group renal ein catheterization was performed.

I each group the effect of exercise was calculated as the significant individual difference of all periods compared to period II tested by Student t-test (Fig. 2—4 and T ble IV) (x $P<0.05$, xx $P<0.01$ xxx $P<0.001$)

Results

Heart rate and blood pressure (Fig I)

The dihydralazine group showed a slightly but consistently higher mean heart rate and mean systolic pressure and lower mean and diastolic pressure than the control group both during and after exercise The post-exercise heart rate was significantly higher ($P<0.01$) in the dihydralazine than in the control group (calculated on the 6 subjects investigated twice)

Urinary findings

The results for both groups are presented as the per cent change of mean values in periods 3—8 compared with period 2 The original values of period 2 are given in Table III

Renal haemodynamics (Fig 2) C_{PAH} decreased significantly during exercise in the control group but not in the dihydralazine group C_{TF} was not significantly decreased in the control group but showed a somewhat more marked decrease in the dihydralazine group which was significant in period 6. Filtration fraction (FF) increased significantly during exercise in the control group but not in the dihydralazine group.

TABLE III. Mean value and standard error of urinary parameters in period 2 (rest before exercise)

Parameter	Mean values and standard error in period 2	
	Dihydralazine group	Control group
C_{PAH} ml/min/1 73 m	598 ± 32	741 ± 39
C_{IN} ml/min/1 73 m	100 ± 3	113 ± 6
FF	16.9 ± 0.7	15.5 ± 1 1
Urine flow ml/min	14.5 ± 1.5	17.5 ± 1.5
C_{H_2O} ml/min	9.9 ± 1 6	12.7 ± 1 1
Urine/plasma inulin ratio	9 ± 2	7 ± 1
C_{osm}/C_{IN} ratio	4.2 ± 0.2	3.6 ± 0.3
Urine Na meq/min	0.278 ± 0.030	0 273 ± 0.035
Urine K meq/min	0.101 ± 0.017	0.125 ± 0.027
% filtered N excreted	1 7 ± 0.2	1.5 ± 0.2
UrineNa/Urine K ratio	3.2 ± 0.5	2.9 ± 0.5

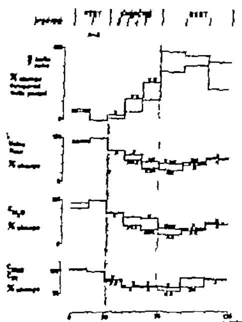


Fig 3.



Fig 4

Fig. 3. Effect of dihydralazine on per cent filtered sodium excreted, urinary sodium (U_{Na}) and potassium (U) excretion, and urine Na/K ratio, during nd after exercise. Per cent change and significant difference of all mean values compared to period 2 are given dihydralazine group (– – –) control group (———)

Fig 4 Effect of dihydralazine on urine/plasma inulin ratio, urine flow C_{H_2O} and C_{osm}/C_{IN} ratio during and after exercise. Per cent change and significant difference of all mean values compared with period 2 are given: *dihydralazine group* (– – –) control group (———)

TABLE IV. Plasma renin activity and P-values for intraindividual differences in periods 5 and 8 compared with period 2.

Subj.	Control group Plasma renin activity ng angiotensin/100 ml plasma			Subj.	Dihydralazine group Plasma renin activity ng angiotensin/100 ml plasma		
	Period 2	Period 5	Period 8		Period 2	Period 5	Period 8
VS	64	190	60	VS	50	400	
SV	99	164		SV	60	90	80
PB	148	674		PB	48	63	350
DW	22	116	17	DW	70	102	562
HW	50	81	90	HW	60	768	
CS	15	147	81	CS	28	100	468
EO	21	62	54	BW	16	62	159
BI	81	270	22	VL	92	804	266
Mean	63	206	56	Mean	53	304	381
P		0.05—0.02		P		> 0.05	0.05—0.02

arterial blood.

TABLE V. Simultaneous determination of plasma renin activity in arterial plasma and renal venous plasma.

Subject	Arterial plasma ng/angiotensin 100 ml plasma Period			Renal venous plasma ng/angiotensin 100 ml plasma Period		
	2	5	8	2	5	8
CS	15	147	81	34	130	
EO	21	62	54	30	207	104
BI	81	270	22	33	197	14
BJ	66		140	6[illegible]		107
Mean	42	160	77	41	178	6

Urine flow (Fig. 3). Urine flow and C_{H_2O} decreased significantly in both groups during exercise most markedly in the control group. The urine plasma inulin ratio increased significantly during exercise in the control group but not in the dihydralazine group. In the first period after exercise C_{H_2O} and urine flow were lower and the urine plasma inulin ratio higher in the dihydralazine group than in the control group.

Electrolyte excretion (Fig 4) Urinary sodium excretion, per cent filtered sodium excreted and urine sodium/potassium concentration ratio decreased significantly in both groups during exercise, most markedly in the dihydralazine group. After exercise these three parameters increased more rapidly towards pre-exercise level in the control group than in the dihydralazine group. Potassium excretion showed a less marked decrease during and after exercise in the dihydralazine group than in the control group.

Plasma renin activity (Table IV and V) The mean plasma renin activity increased in both groups during exercise, most markedly in the dihydralazine group (Table IV). Because of the wide individual variation this increase was only significant in the control group Using the Wilcoxon matched pairs signed-ranks test there was a significant increase ($P<0.05$) also in the dihydralazine group. The post-exercise plasma renin activity increased further significantly compared with the resting level in the dihydralazine group but returned to the pre-exercise level in the control group. Plasma renin activity increased also in renal venous plasma during exercise (Table V) The mean values indicate parallel changes in peripheral arterial and renal venous plasma.

Discussion

Dihydralazine had a clearly inhibitory effect on the decrease in C_{PAH} caused by exercise. Its effect on C_{IN} during exercise was less marked but the mean value was slightly and consistently lower These two effects contribute to the almost complete inhibition of the normal increase in FF during exercise The renal hemodynamic response may be partly caused by a vasodilatory effect on the afferent and the efferent glomerular vessels. The central hemodynamic effects of intravenous administration of dihydralazine in man during rest were studied by Åblad (1963) He states that the peripheral vasodilatory effect of hydralazine causes a reflex increase in the sympathetic vasoconstrictor fibre discharge. The increased heart rate and systolic pressure and decreased diastolic pressure in the dihydralazine group compared with the control group in this study are in agreement with his results.

The factors controlling the secretion of renin from the juxtaglomerular apparatus are poorly understood. Several hypotheses have been proposed. The stimulus for renin release has variously been considered as renal ischemia (Houssay and Braun-Menendez 1942) a change in pulse pressure (Kohlstaedt and Page 1940) stretch (Tobian 1964) and mean pressure (Skinner *et al.* 1964) of the afferent glomerular artery Changes in proximal intratubular pressure have also been proposed as regulating the renin release (Leyssac 1964) Others have suggested that a change in the sodium concentration (Thurau 1964) or sodium load (Wander 1965 Gross, Brunner and Ziegler 1965) or osmolality (Brown *et al.* 1964) in the distal tubules in close proximity to macula densa is the stimulus to renin release.

Plasma renin activity was significantly increased during exercise in the normal untreated group which is in accordance with the increased plasma renin activity

after prolonged heavy exercise (Božović, Castenfors and Piscator 1967) There is also an increased plasma renin activity in renal venous plasma during exercise. This cannot be attributed to the effect of renal vasoconstriction because, in the dihydralazine group when the vasoconstriction was partially inhibited, an even more marked mean increase in plasma renin activity was found. After exercise the plasma renin activity returned to normal in the control group but increased further in the dihydralazine group in spite of the increased renal plasma flow In the dihydralazine group increased pulse pressure and stretch of the afferent glomerular artery according to the above-mentioned theories should inhibit renin secretion. The decreased mean pressure in the dihydralazine group compared with control groups may be expected to stimulate renin release. In the study of Skinner *et al.* (1964) the mean arterial pressure had to be lowered about 5—10 mm Hg to stimulate renin release, but the difference between the two groups in this study was only 3 mm Hg in the last period of rest after exercise, when the most marked difference in plasma renin activity was recorded. Constriction of afferent glomerular arterioles and primary increase in proximal tubular sodium reabsorption are considered to reduce the proximal intratubular pressure and stimulate the production of angiotensin (Leyssac 1964) The significantly increased plasma renin activity in period 8 in the dihydralazine group is difficult to explain according to the expected changes in proximal intratubular pressure. The stimulus for increased renin secretion during exercise does not seem to be dependent on an intrarenal baroreceptor according to the above mentioned theories.

Dihydralazine may have a direct stimulatory effect on renin release However studies on rats (Božović and Castenfors, 1967) ha e shown that ganglionic blocking can prevent the stimulatory effect of dihydralazine on renin release, which indicates that the latter seems to be mediated by a nervous mechanism.

The glomerular vascular pole where the juxtaglomerular apparatus is located has a rich innervation which is presumed to be sympathetic (Barajas 1964) Taquini *et al* (1964) demonstrated that denervation of rat kidneys caused a decrease in thei renin content. This suggests that the sympathetic nervous system may play an important role in the control of renin secretion A striking hemodynamic effect of hydralazine is the increased sympathetic stimulation mentioned earlier It is suggested that the stimulus for renin secretion in this study is an increased sympathetic stimulation acting directly on the juxtaglomerular apparatus, irrespective of its renal hemodynamic effect. This is in agreement with the finding that catecholamine infusion into the renal artery (Wathen *et al* 1965) or i catecholamine infusion or stimulation of the renal nerves increased the renin release in anesthetized dogs (Vander 1965) In the study of Vander the renin release was eversed or prevented by induction of osmotic diuresis and was therefor uggested to be secondary to the decrease in filtered sodium. The tendency to decreased C_{PAH} and urinary sodium excretion in the dihydralazine group compared with the control group may support this interpretation. However a marked ncrease in sodium excretion during exercise induced by intravenous ethacrynic acid did not reverse but further increased the renin release during

exercise (Castenfors 1967b) Different species and experimental conditions may explain the contradictory results.

The effect of dihydralazine on renal handling of water was different during and after exercise. Differences in medullary circulation and/or ADH release may be an explanation, but the small differences between the two groups do not allow evaluation of these factors. This study was not designed for that purpose.

During exercise normal subjects show a significant positive correlation between renal plasma flow and urinary sodium excretion (Castenfors 1967a) The less marked decrease in renal plasma flow in the dihydralazine group would be likely to minimize the decrease in the urinary sodium excretion. Dihydralazine, however caused a slightly more marked decrease in mean urinary sodium excretion than in the control group, which suggests the presence of a potent sodium retention factor Aldosterone injected intravenously has a latent period of more than 30 min. This suggests that the possible increase in aldosterone secretion secondary to increased plasma potassium and plasma renin activity may only partly contribute to the sodium retention at the end of and after exercise. Some authors, Fischer and Takács (1959) and Fekete (1959) claim that nervous mechanisms participate directly in the regulation of sodium reabsorption in the tubules. It cannot be excluded that the rapid decrease in sodium excretion during exercise may be secondary to such a mechanism.

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Acta physiol. scand. 1967 70 290—292

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Effect of Ganglionic Blocking on Plasma Renin Activity in Exercising and Pain-Stressed Rats

By

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Abstract

BOŽOVIĆ, L. and J CASTENFORS. *The effects of ganglionic blocking on plasma renin activity in exercising and pain-stressed rats* Acta physiol scand. 1967 70 290—292

The effect of ganglionic blocking on plasma renin activity and plasma sodium concentration during exercise and pain stress were studied in rats. 20 female albino rats were exercised by swimming and 16 received intramuscular injections of 1 ml 10% NaCl. Half of the animals in each group were pretreated with ganglionic blocking drug (Ansolysen ®) In both groups there was significantly lower plasma renin activity in the ganglion-blocked animals compared with their untreated controls, which indicates the importance of the sympathetic system for renin release. The findings suggest that changes in plasma sodium concentration are of minor importance compared with sympathetic activity

A previous study (Božović and Castenfors 1967) indicates that plasma renin activity increases during exercise in man and suggests that the sympathetic system may have a direct effect on renin release, irrespective of its intra-renal hemodynamic effect.

The present investigation was undertaken to determine whether ganglionic blocking inhibits renin release in exercising and pain-stressed rats. Both procedures are known to increase sympathetic activity

Material and method

Thirty-six female albino rats, weight 200—250 g, were investigated. *Sodium and potassium* were determined in plasma and urine by flame photometry

The hematocrit was measured with the International Haematocrit Centrifuge (11,000 r p m for 5 min)

Plasma renin activity was determined according to the method of Boucher (1964) with the modification that heparin was used as anticoagulant instead of EDTA. Two ml of rat plasma was added to 5 ml saline and further processed by the method of Boucher Renin activity was expressed as ng angiotensin/100 ml plasma, formed after 3 hrs incubation at 37 °C.

Procedure

Exercise. 10 rats were injected intraperitoneally with pentolinium (Ansolysen ®) 2 mg/100 g 1 hr before the test. The exercise consisted of swimming in water at temperature of approximately 35 C.

Ganglionic blocking markedly decreased the ability to swim Exercise was performed in such a way that the animal swam until exhaustion (dipping of the head twice under water) and was then

taken out and allowed to rest for three minutes, whereafter the procedure was repeated. The entire procedure took 45 min and the average swimming time was about 25 min. The time scale for each rat was repeated with an untreated control animal, which thus performed the same amount of exercise. At the end of the exercise the animal was anesthetized with pentothal intraperitoneally the abdomen was opened surgically and blood sample was immediately taken from the abdominal aorta by direct puncture.

Pain stress Eight rats were injected intraperitoneally one hour before the experiment with pentolinium, 2 mg/100 g, 8 rats were untreated. Pain was induced during 1 hr by i.m. injection of 0.5 ml 10% NaCl at the start and again after 30 min. Blood was collected in the same way as in the exercise experiment.

Results (Table I)

Exercise There was a significantly lower plasma renin activity in the pentolinium group compared with the control group. Plasma sodium was lower in the pentolinium group than in the control group but the difference was not statistically significant.

Pain stress Plasma renin activity was significantly lower in the pentolinium-treated group compared with the control group. The plasma sodium concentration was the same in the two groups.

TABLE I Effect of ganglionic blocking on plasma renin activity* and plasma sodium concentration in exercising and pain-stressed rats

Parameter	Exercise, swimming			Pain, 1 ml 10% NaCl i.m.		
	Control group	Sign. diff. P	Pentolinium group	Control group	Sign. diff. P	Pentolinium group
Number of rats	10		10	8		8
Plasma sodium meq/l	131.4 ± 1.7	>0.05	124.1 ± 3.6	143.1 ± 2.3		144.2 ± 2.2
Plasma renin activity ng angiotensin/100 ml plasma	1,370 ± 197	<0.02	700 ± 103	1,790 ± 315	<0.01	570 ± 121

* Normal resting value about 500 ng/100 ml (unpublished observations)

Discussion

The significantly lower plasma renin activity in the ganglion-blocked swimming and pain-stressed animals compared with their controls verifies the importance of sympathetic activity for stimulation of renin release.

The high plasma renin activity and high plasma sodium value during exercise in the control group compared with the ganglionic blocked group do not agree with the findings in man (Brown *et al* 1966) that plasma renin concentration correlates negatively to plasma sodium concentration.

The pain-stressed animals were subjected to severe sympathetic stress and were also markedly loaded with sodium. Treatment with 1 ml of 10% NaCl added about 1.7 meq of sodium, which amounts to about 30 per cent of the extracellular sodium in the rats. The high plasma sodium concentration in both groups, compared with the

exercising rats, indicates sodium loading. The significant difference in plasma renin activity in the absence of significant changes in plasma sodium concentration, between the ganglion blocked pain-stressed rats and their controls emphasizes the role of the sympathetic nervous system in effecting renin release. Under these experimental conditions changes in plasma sodium concentration cannot be the determining factor in renin release.

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The Effects of Varying the Extracellular Potassium Concentration on the Secretory Rate and on Resting and Secretory Potentials in the Perfused Cat Submandibular Gland

By

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Abstract

Petersen, O. H. and J. H. Poulsen. *The effects of varying the extracellular potassium concentration on the secretory rate and on resting and secretory potentials in the perfused cat submandibular gland.* Acta physiol. scand. 1967. 70. 293—298.

Lundberg presented the hypothesis that the secretory potentials in salivary glands were due to an active transport of chloride ions. This transport should be responsible for the formation of the saliva. Recently a different hypothesis has been proposed by Imai, who postulated that the secretory potentials were caused by an increased permeability of the cell membranes to potassium ions. The present investigation was undertaken to test Imai's hypothesis. It was found that the secretory potentials and the secretory rate depended in very much the same way on the extracellular potassium concentration. This gives further support to Lundberg's hypothesis.

Lundberg (1955, 1957a) recorded transmembrane potentials in cat submandibular and sublingual gland cells. It was shown that the transmembrane potential in the acinar cells of the submandibular gland and the corresponding tubular cells in the sublingual gland (a tubular gland) hyperpolarize when stimulating the gland. This hyperpolarization was named the secretory potential. In the sublingual gland Lundberg (1957b), using double-barrelled microelectrodes, showed that the secretory potential was independent of the size of the resting potential in the range from −10 to −100 mV. Lundberg (1957c) further showed that both secretion and secretory potential was highly dependent on the presence of chloride ions in the perfusion fluid. It was therefore concluded that the secretory potentials were due to an active transport of chloride ions into the cells through the contraluminal cell membrane. This view was supported by Petersen and Poulsen (1966) who showed that acetazolamide, which inhibits active chloride transport in a number of secretory organs (Davson 1964), inhibits both secretory potentials and secretory rate in the submandibular gland of the cat.

Burgen (1956) showed that salivary glands during the first period of secretion lose potassium to the saliva as well as to the blood.

Imai (1965) recorded secretory potentials in the perfused dog submandibular gland. When the potassium concentration in the perfusion fluid was elevated, the secretory potentials diminished and were completely abolished at potassium concentrations above 13 mmoles/l. At these concentrations some secretion was still present. When potassium was omitted from the perfusate, the secretion stopped, while "almost normal" secretory potentials could still be recorded. As the resting acinar membrane potential is less than the potassium equilibrium potential, Imai (1965) concluded that the secretory potentials were due to an increased permeability of the cell membranes to potassium.

As Imai's (1965) findings are incompatible with Lundberg's (1957b) experiments, it was decided to reinvestigate the effects of different extracellular potassium concentrations on secretory rate, resting and secretory potentials in the perfused cat submandibular gland. The result that the size of the secretory potentials and the secretory rate depended in very much the same way on the extracellular potassium concentration is fully compatible with Lundberg's (1958) hypothesis, while it gives no support to Imai's (1965) theory.

Methods

Young cats weighing 1—3 kg were anaesthetized with chloralose (70—90 mg/kg intraperitoneally). The left submandibular gland and its duct was prepared as described by Lidell and Sherrington (1929). Briefly described, the gland was exposed, stimulating electrodes were placed on the lingual nerve and the submandibular duct was cannulated with thin polyethylene tube.

Preparation for artificial perfusion

The common carotid artery on the left side was traced and cleaned, and all its branches except the one supplying the submandibular gland were ligated. The external jugular vein was traced and some of the biggest tributaries not draining the submandibular gland were ligated. I order to be able to measure the glandular saline flow polyethylene tube was inserted into the external jugular vein. Finally polyethylene tube was inserted into the common carotid artery. Through this cannula the gland was perfused with normal and modified Locke solutions, under non-pulsatile (hydrostatic) pressure of 90 mm Hg. As soon as the gland was being perfused, the animal was killed by bleeding from the left femoral artery.

The perfusion fluids

The principal perfusion fluid used was a Locke solution with the following composition (mmoles/l): Na 142, K 4.0, Ca 1.5, Mg 1.0, Cl 127, HCO^- 22, HPO 1.1, glucose 10. Modified Locke's solutions with the following potassium concentrations were used: 0.2, 0.3, 20, 30 mmoles/l. In these cases corresponding changes in the sodium concentration ensured constant osmolarity. The perfusion fluids were equilibrated with 5—6 CO in O. I order to increase the mount of dissolved oxygen and to lower oxygen consumption of the gland, the perfusion was carried out at room temperature.

Stimulation of the gland

Secretory potentials were evoked partly by electrical stimulation (10 c/sec, 10 V) of the chorda-lingual nerve, and partly by injection of acetylcholine (5 µg) in a thin polyethylene cannula situated inside the common carotid cannula.

For the experiments with measurement of the secretory rate continuous infusion of acetylcholine (70 µg/min) was used.

Measurement of transmembrane potentials

Conventional glass capillary micropipettes were draw by Palmer microelectrode puller. The micropipettes were filled with 3 M NH_4NO_3 by boiling under reduced pressure after heating to

temperature of 80—85 C. Only electrodes having impedances from 15 to 50 MΩ were used. The tip potentials wer always less negative than —5mV (usually less than —3mV) The electrodes were connected to a cathode follower (grid current less than 10^{-13} A) The cathode follower was connected to a DISA dc-amplifier The amplified signal was localized on a DISA oscilloscope screen, and recorded with Mingograf writer The microelectrode was mounted on a de Fonbrune pneumatic micromanipulator and impaled in small decapsulated area of the gland under visual control through binocular Zeiss dissecting microscope (20 × magnification) Potentials were never recorded until t least 5 min (generally 10 min) had elapsed after changing the perfusion fluid.

Measurement of secretory rates

From the duct cannula the saliva was collected into tuberculine syringe. The secretory rat was measured in the first minute of secretion. The secretory rate was measured from 10 to 15 mi after each change of the perfusate.

Results

All the secretory potentials in the present work belong to Lundberg s (1955) type I group, originating from the acinar cells.

The dependence of the resting and secretory potentials on the extracellular potassium concentration is shown in Fig 1 Examples of secretory potentials at different potassium concentrations are shown in Fig 2.

Fig. 3 shows typical examples of how the secretory rate in single experiments depends on the potassium concentration.

In Fig 4 a comparison between the dependence of the secretory potentials and the secretory rates on the potassium concentration is shown.

At a potassium concentration of 50 mmoles/l, where it was very difficult to obtain secretory potentials (the secretion nearly stopped at this concentration) few secre

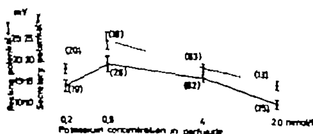


Fig. 1 The size of resting and secretory potentials as func tion of the potassium concentration in the perfusate. Resting potential ○——○ secretory potential ——× Mean sizes ± S.E. nd number of measurements (in parentheses) are indicated for the four different potassium concentrations used

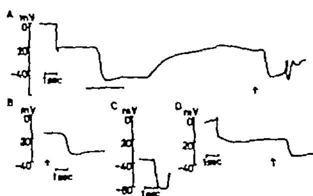


Fig. 2. Examples of secretory potentials at different potassium concentrations. Chorda stimulation ——— acetylcholine injection ↑ A Resting and secretory potentials at potassium concentration of 4 mmoles/l. The secretory potentials shown are considerably greater than the mean B Secretory potential at potassium concentration of 0 2 mmole/l. C Secretory potential potassium concentration of 0 5 mmole l D Resting and secretory potential at potassium concentration of 20 mmoles/l.

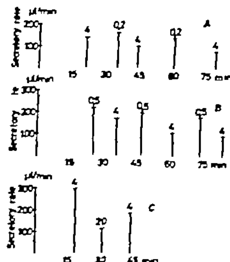


Fig. 3. Secretory rates at different potassium concentrations in 3 typical experiments. The numbers above the columns indicate the potassium concentration in the perfusate



Fig. 4. A comparison of the sizes of the secretory potentials and the secretory rates at different potassium concentrations in the perfusate. The values are given in per cent of the mean values obtained at potassium concentration of 4 mmoles/l ± S.E. Secretory rate – – – ○ secretory potential: × —— ×

tory potentials with a size of about 5mV were recorded. In a single cell a resting potential as large as —22 mV was measured. This cell hyperpolarized with 5 mV during stimulation.

Discussion

It was originally demonstrated by Burgen (1956) that salivary glands during the first period of secretion lose potassium to the saliva as well as to the blood (the so-called potassium transient). At least 3 different mechanisms for the potassium transients could be proposed. 1) The cell membranes increase their permeability to potassium when the gland is stimulated. As the resting transmembrane potential is about —20 mV (Lundberg 1955, Poulsen and Petersen 1966) in the cat submandibular gland and —42 mV in the dog submandibular gland (Imai 1965) and as the potassium equilibrium potential is about —90 mV (Burgen and Emmelin 1961) the increased permeability would lead to diffusion of potassium out of the cells and to a hyperpolarization of the cell membrane. 2) The potassium ions are pumped out of the cells by an active mechanism. 3) The transport of potassium is due to a solvent drag effect.

If possibility 1) was the right explanation, one would expect changes in the size of the hyperpolarization (the secretory potential) with changes in the potassium concentration (caused by changes in the potassium equilibrium potential) without corresponding changes in the secretory rate. In the present experiments, secretory potential and secretory rate varied in the same manner (Fig 4) In opposition to this Imai (1965) in his experiments in dogs found changes in the size of the secretory potentials following changes in the potassium equilibrium potential without corresponding changes in the secretory rate. The result presented in Fig 1 that the sensitivity of resting potentials and secretory potentials to changes in the potassium concentration is the same, is incompatible with Imai's (1965) hypothesis that the secretory potentials are due to an increased permeability of the cell membrane to potassium. If Imai's (1965) hypothesis were correct, the secretory potentials should be more influenced by variations in the potassium concentration than the resting potentials. Another argument against Imai's (1965) hypothesis is that both resting and secretory potentials decrease when the potassium concentration is changed from 0.5 to 0.2 mmole/l. (Fig 1) Furthermore Lundberg (1957b) has shown that secretory potentials can still be evoked at resting potentials greater than the potassium equilibrium potential. Finally our finding in one single cell, that at an extracellular potassium concentration of 30 mmoles/l (potassium equilibrium potential = −21.4 mV using Imai's (1965) value for the intracellular potassium concentration of 117 mmoles/l) a secretory potential of 5 mV could be evoked at a resting potential of −22 mV fits well with Lundberg's findings.

Possibility 2) seems unlikely

Possibility 3) that the loss of potassium from the gland cells should be due to a solvent drag effect suits the hypothesis of Lundberg (1958) very well. According to this hypothesis the first step in the formation of saliva is an active, electrogenic transport of chloride ions through the contraluminal acinar cell membrane into the cell As a consequence of this electrogenic transport, which increases the intracellular negativity cations (mainly sodium) are passively transported into the cells. This will increase the intracellular osmolarity Therefore water diffuses into the cells, thereby increasing the intracellular pressure This causes a filtration through both the luminal and the contraluminal cell membrane If this model is true, a solvent drag effect may be exerted on the potassium ions, causing a transport of potassium from the cells into both saliva and extracellular space This model for transepithelial transport of fluid in salivary glands is essentially the same as Diamond's (1964) more recent models (local osmosis and double membrane effect) for transepithelial fluid transport in the gall bladder

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Acta physiol. scand. 1967 70 299—311

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Sympathetic Control of Rhythmically Active Vascular Smooth Muscle as Studied by a Nerve-muscle Preparation of Portal Vein

By

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Abstract

Johansson B. and B Ljung *Sympathetic control of rhythmically active vascular smooth muscle as studied by a nerve-muscle preparation of portal vein.* Acta physiol. scand. 1967 *70* 299—311

Techniques are described for recording directly the contractile responses elicited in the longitudinal smooth muscle of cat or rabbit portal vein by vasomotor nerve stimulation in situ or in vitro. This vascular smooth muscle, which shows spontaneous rhythmic activity of 3 to 8 contractions per minute responded to splanchnic or postganglionic nerve stimulation by an increase in the mean level of tension associated with an increased frequency of contractions Guanethidine abolished these neurogenic responses indicating that they were due to adrenergic sympathetic fibre activation. Effects of vagal nerve stimulation were weak and inconstant. The quantitative tension responses produced by splanchnic or postganglionic nerve stimulation at graded impulse rates increased steeply in the low frequency range and reached maximum at 8 to 16 imp/sec. These frequency-response curves, obtained by direct recording of vascular smooth muscle contractions, are in conformity with those found in previous investigations studying blood flow or regional blood volume in intact vascular beds. The mechanisms by which the sympathetic innervation controls activity in rhythmically active vascular smooth muscle and the quantitative aspects of this influence are discussed in the light of the present results.

The range of control exerted by the autonomic nervous system on vascular smooth muscle has been investigated chiefly with indirect methods measuring blood flow (e.g. Folkow 1952, Celander 1954) or changes in regional blood volume (e.g. Mellander 1960) More detailed information on the mode of action of the vasomotor nerves upon the vascular effectors would probably be obtained if the responses could be studied more directly Bevan (1962) has utilized an isolated sympathetic nerve-pulmonary artery preparation for studying neuromuscular function in this particular vessel. The response of pulmonary artery smooth muscle to the adrenergic transmitter is a slow contraction which appears to be independent of electrical events

A preliminary report of the present study was given at the XIIth Scandinavian Physiology Congress, Turku 1966 (Acta physiol scand. 1966, 68, Suppl. 277 94)

at the cell membrane (Su, Bevan and Ursillo 1964) Smooth muscle in the hemodynamically important resistance vessels of the systemic circulation is quite different in its functional characteristics as indicated by its rhythmic contractile activity which is probably myogenic in nature (*e g* Chambers 1948, Folkow 1964 Johansson and Bohr 1966) Such myogenic activity is present also in portal and mesenteric veins where the phasic contractions have been shown to be correlated with propagated action potentials (Funaki and Bohr 1964 Cuthbert and Sutter 1964 Axelsson *et al.* 1966) It was considered of interest to find out in what way vasomotor nerve fibre activity modulates basal rhythmicity and contractile force in vascular smooth muscle of this propagating type. Techniques are described in the present report which allow direct recording, in situ or in vitro, of the tension responses elicited in the innervated cat or rabbit portal vein by stimulation of the vasomotor nerve supply Quantitative aspects of neuromuscular function in vessels are discussed in the light of the results obtained with these preparations.

Material and methods

Contractile responses of the portal vein in situ were recorded in experiments performed on 10 rabbits and 14 cats. I 13 experiments isolated nerve-muscle preparation of rabbit portal vein with intact postganglionic nerve supply was studied in vitro.

In situ experiments. The animal preparation, illustrated schematically in Fig 1 was in principle the same for rabbits and cats. The animals were anaesthetized with pentobarbital, 30 mg/kg i The left femoral vein was cannulated for injections. A tracheal cannula was inserted to insure free respiratory pathways and permit artificial ventilation. The vagi were cut bilaterally and the peripheral ends were placed on bipolar stimulating electrodes. The left femoral artery was cannulated for blood pressure recording using a Statham pressure transducer type P23 AC.

The abdomen was opened by midline incision. The splanchnic nerves were dissected and cut bilaterally and the distal ends were fitted into ring-shaped insulated bipolar silver electrodes. T eliminate the influence of adrenomedullary hormones, released on splanchnic nerve stimulation, the adrenal glands were tied off bilaterally the loss of corticosteroids being substituted by intramuscular injection of hydrocortisone, 5 mg/kg

The intestines and the antrum of the stomach were extirpated without interfering with the major veins draining blood from stomach and spleen into the portal vein. The peritoneal layer covering the portal vein was split and the vascular wall was partly mobilized by gentle dissection. One of the larger branches of the vein entering the liver was tied nd cannulated with an L-shaped plastic tube and this was connected by rubber tubings to cannula in the right jugular vein. After this shunt had been applied the remaining portal branches were liga ed in the hepatic hilus so that portal flow now bypassed the liver entirely Blood flow and pressure in the portal vein would then be independent of changes in intrahepatic ascular tone I 5 of these experiments an optical drop flow recorder (Lindgren 1958) was introduced into the shunt circuit. A pressure transducer

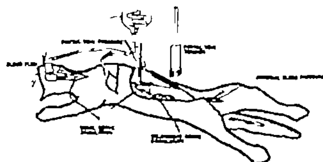


Fig 1 Schematic illustration of the preparation used for recording tension responses of the innervated portal vein in situ. The hepatic end of the vessel as cannulated and immobilized, the mesenteric end liga ed and connected to force-displacement transducer Arterial blood pressure portal venous outflow pressure and portal blood flow were recorded together with portal vein tension

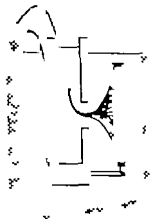


Fig. 2. Schematic drawing of muscle holder and electrode arrangement used for stimulating postganglionic nerves to the portal vein in itro. One end of the muscle preparation was anchored to the plastic holder the other connected to the tension transducer. The nerve bundle was pulled through hole in rubber membrane into the central chamber of the plastic holder. Note the electrodes in inner and outer bath.

Statham P23 BC, was connected to side-branch of the shunt for recording of portal venous outflow pressure.

The L-shaped cannula at the hepatic end of the portal vein was anchored to rigid metal rod whereas the mesenteric end, which had been ligated on extirpation of the intestines, was mobilized nd ttached to force-displacement transducer (Grass FT 03). Mechanical artifacts due to respiratory movements were eliminated by making broad thoracotomies and by cutting the phrenic nerves bilaterally. The animals were artificially ventilated at tidal volumes small enough for the lungs barely to reach the diaphragm. This was possible without obvious signs of hypoventilation. The external environment of the portal ein was kept constant by continuous superfusion with physiological salt solution (see below) bubbled with 4 per cent CO_2 in O and kept at temperature of 37 C. Excess fluid was removed from the abdominal cavity by continuous suction. Drugs could be applied locally by adding them to the superfusion fluid.

The isometric tension responses of the longitudinal musculature of the portal vein, reflected in the electrical output of the force-displacement transducer were recorded together with arterial and portal pressure on the Grass polygraph. Portal enous outflow was recorded on smoked drum by an ordinate writer operated by the optical drop counter and giving ordinate heights inversely propor tional to the rate of flow. A Grass stimulator type S5 was used for nerve stimulation.

I was often difficult to keep the rabbits in good condition for longer experimental periods but the cats usually maintained good cardiovascular state and responsiveness for hours despit the extens preparation.

In itro experiments. Rabbits were killed by blow over the neck. The bdomen was opened and the nerv branches running from the right coeliac ganglion towards the portal ein were dissected and cut centrally. Also the portal vein was freed from peritoneum, ligated at both ends and cut. The retroperitoneal tissue on the left side of the ein was cut carefully leaving block of tissue ith the portal ein and the postganglionic fibres of the right side. The vein was then cut open and an innervated strip measuring pproximately 15 3 mm was prepared. The nerve-muscle preparation as placed in mantled organ-bath where the physiological salt solution was continuously bubbled and kept at 37°C. One end of the muscle trip was fixed to plastic holder (Fig. 2) and the other end attached to force-displacement transducer (Grass FT 03). The nerve bundle was pulled through hole made in thin rubber membrane on the muscle holder which functioned as fluid electrode similar to that used by Garry and Gillespie (1955). The stimuli delivered to the nerve ere monitored on an oscilloscope connected in parallel with the stimulator (Grass S5). Some short-circuiting around the nerv was inevitable. The muscle strip was stretched to passiv tension of 400 to 800 dynes gi ing it approximately in situ length. The preparation was allowed to accommodate for one hour before the experiment was started.

Motor responses of the vascular smooth muscle to nerve stimulation and to drug exposure were quantitated in both in vivo and in vitro experiments in the following way: The area under the curve recorded during the minute preceding the stimulating period and that obtained in the first minute of stimulation were cut out from paper and weighed. Knowing the paper weight per unit area it is possible to calculate the increase in mean tension developed during the first min of stimulation.

The physiological salt solution used in all experiments had the following composition in mmoles/l: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 14.9, dextrose 5.5, sucrose 50, $CaCl_2$ 1.6, and calcium disodium versenate 0.026.

The following drugs were used: l-noradrenaline (Norexadrine, Astra), dl-isoproterenol sulphate, acetylcholine chloride, phenoxybenzamine-HCl (Dibenzyline, Smith Kline & French), guanethidine disulphate (Ismelin, CIBA), atropine sulphate and synstigmine methyl-sulphate (Neostigmine, Leo). Drug-concentrations are expressed in weight/volume calculated as the base for noradrenaline and as the respective salt for the other drugs.

Results

A. In vivo experiments

The "spontaneous" rhythmic variations in tension typical of the longitudinal muscle of the portal vein is shown between the stimulation periods in Fig. 3 and 4. This activity, which was essentially the same in cats and rabbits, consisted of three to eight major contraction waves/min with variable regularity and amplitude. As a rule there was no constant baseline between the phasic contractions indicating that relaxation was not complete before the next contraction started.

Fig. 3, which illustrates the effects of graded stimulation of the splanchnic nerve is taken from one of the experiments where portal venous outflow was recorded together with arterial blood pressure, portal vein tension and portal venous outflow pressure. Each period of splanchnic nerve stimulation caused an increase in arterial blood pressure and a reduction in blood flow due to constriction of resistance

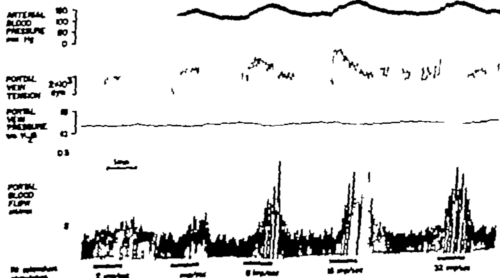


Fig. 3. Cat 3.7 kg. Pentobarbital. Effects on arterial blood pressure, longitudinal portal tension, portal venous outflow pressure, and portal blood flow produced by stimulating the right splanchnic nerve with impulses of 4 msec and 15 V at graded frequencies.

vessels in the stomach and the spleen. Blood flow did not decrease during the first 10 to 15 sec of stimulation but an initial increase in venous outflow due to an active expulsion of blood from the capacitance vessels, was often noted instead.

The recording of portal vein tension in Fig. 3 displays a tendency towards greater amplitude and irregularity of the contractions with nerve stimulation at 2 imp/sec compared to control. A more clear-cut increase in frequency of the major contractions and an overall increase in tension occurred at 4 and 8 imp/sec. This increase became more marked with 16 and 32 imp/sec but the responses to these higher frequencies were not sustained. The portal venous outflow pressure was relatively constant throughout the experiment: the slight variations that occurred were related mainly to the rate of blood flow through the vein. Therefore, the longitudinal tension responses induced in the portal vein were not passive effects due to changes in pressure but resulted from active muscle contraction. Since the adrenal glands were tied off in the experiment of Fig. 3 the portal vein responses can be ascribed to activation of vasomotor nerve fibres supplied to the portal vein (see further below). Portal vein responses like those illustrated in Fig. 3 were induced by stimulation of both right and left splanchnic nerves without any obvious difference between the two sides. Simultaneous stimulation of both nerves gave responses that were not significantly larger than those obtained by unilateral stimulation.

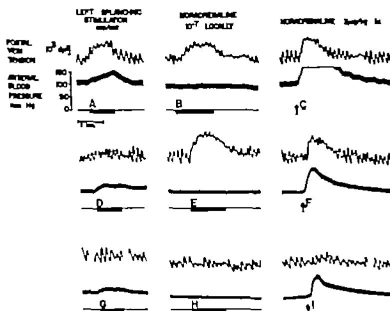


Fig. 4. Cat 3.6 kg. Pentobarbital. Responses of portal vein tension and arterial blood pressure produced by left splanchnic nerve stimulations at 4 imp/sec, 4msec and 15 V (A, D and G) by local applications of noradrenaline 10- on the portal vein (B, E and H) and by i.v. injections of noradrenaline 3 µg/kg (C, F and I). Note that the portal vein response to splanchnic stimulation is abolished by guanethidine 10 applied on the vein between C and D and that the responses to noradrenaline are eliminated by phenoxybenzamine 10 applied locally between F and G. The systemic effects are not interfered with by the topical drug applications.

That the contractile responses of the portal vein to splanchnic nerve stimulation is a direct effect of adrenergic innervation is shown by the experiment illustrated in Fig 4 This demonstrates changes in portal vein tension and arterial blood pressure in response to left splanchnic nerve stimulation, to local application of noradrenaline on the portal vein and to i.v injection of noradrenaline. In the control situation, stimulation of the splanchnic nerve at 4 imp/sec (A) local application of noradrenaline, 10^{-} in the solution superfusing the portal vein (B) and i.v injection of noradrenaline, 3 µg/kg body weight (C) all gave tension responses in the portal vein of approximately equivalent amplitude. Arterial blood pressure increased in A and C but no systemic effect was seen with the local noradrenaline administration in B The experimental procedures were repeated after the vein had been exposed for 3 min to guanethidine, 1 mg/l in the superfusion solution. After guanethidine the portal vein response to splanchnic nerve stimulation was abolished (D) whereas local and systemic administration of noradrenaline still caused contraction of the vein (E, F) Arterial blood pressure decreased in the course of the experiment but the pressor responses to the three experimental procedures were the same in principle as in the control period. Local application of the alpha-adrenergic blocking agent phenoxybenzamine in a concentration of 1 mg/l completely blocked the portal vein responses without altering the systemic blood pressure effects (G H, I) The spontaneous rhythmic contractions of the portal vein were not changed significantly by guanethidine or phenoxybenzamine in the concentrations used. It is further notable that splanchnic nerve stimulation failed to cause either activation or inhibition of the portal vein after alpha-adrenergic blockade.

The quantitative correlation between the rate of stimulation of the splanchnic nerves and the response of the smooth muscle of the portal vein, as exemplified in Fig 3 was studied in a total of 10 experiments. The results of these are summarized in the diagram of Fig 5 The voltages and impulse durations used can be expected to have given a supramaximal stimulation of the sympathetic preganglionic fibres in the splanchnic nerves. The responses to the different impulse frequencies were quantitated as described above and were then, in each series of stimulations, expressed

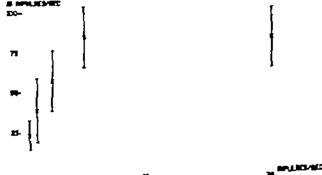


Fig. 5 Diagrammatic representation of mean tension responses obtained in 10 expts on cat portal vein in situ during supramaximal splanchnic nerve stimulation at different impulse frequencies Responses expressed as per cent of response to 16 imp/sec. Mean values ± S.D. are indicated.

in per cent of the response to 16 imp/sec. Mean values ± S D are represented in Fig 5 The responses increased steeply in the low frequency range to reach a maximum at 8 or 16 imp/sec whereas a reduction in response occurred at 32 imp/sec. This latter phenomenon was even more pronounced in the few experiments where still higher frequencies were used.

It appears from the recordings in Fig 3 and 4 that splanchnic nerve stimulation increased tension of the portal vein partly by a positive chronotropic effect i e. by increasing the frequency of the phasic contractions. Due to the irregularity of the activity of the in vivo preparation it was not possible to quantitate this chronotropic action separately

As mentioned above portal venous outflow originating mainly from stomach and spleen, was recorded in five experiments of which one was represented in Fig 3 After the initial 10—15 sec, where the resistance response was masked by emptying of blood from the veins, the blood flow recording indicated constrictions of the resistance vessels that were quantitatively related to the frequency of splanchnic nerve stimulation. Despite the limited number of experiments and the variations observed it may be said that these responses, expressed in per cent of maximum, show a relationship to stimulation frequency similar to that given for the portal vein responses in Fig 5

Stimulation of the right or left vagal nerves at 1—20 imp/sec, 4 msec and 10 V gave a slowing of the heart and a fall in blood pressure but no consistent changes in portal vein activity There was, however in some experiments a slight increase in the vein tension Local application of acetylcholine in concentrations of 1 mg/l in the solution superfusing the portal vein produced an increase in the tension level which was abolished by application of atropine. The slight and inconsistent tension increases produced by vagal nerve stimulation were not clearly eliminated by atropine or phenoxybenzamine nor were they enhanced by local application of synstigmine.

B In vitro experiments

Several of the isolated preparations of rabbit portal vein showed a spontaneous rhythmicity quite similar to that of the vessel in situ. However in some of the in vitro experiments there was a slightly different pattern of spontaneous activity consisting of distinctly separated phasic contractions starting from a constant

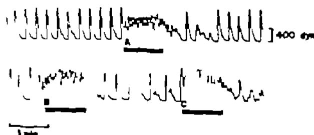


Fig 6 Responses of innervated strip of rabbit portal vein to postganglionic nerve stimulation in vitro. A 4 imp/sec, B 8 imp/sec C 16 imp/sec Note the increase in contraction frequency produced by nerve stimulation in this regularly contracting muscle

base line (Fig 6) This base-line represents the passive resting tension of the muscle as indicated by the fact that its level was not changed by isoproterenol in concentrations which completely inhibited the phasic contractions. This pattern of regular activity where the preparation behaved as a true "single unit muscle" was observed predominantly in experiments on relatively narrow (2—3 mm) strips of portal vein. The influence of nerve stimulation on the frequency of contraction, "the chronotropic effect" is most clearly seen in recordings from such experiments.

Fig 6 illustrates responses to stimulation of the postganglionic fibres in the isolated nerve-muscle preparation. An approximately four-fold increase in contraction frequency is seen at 4 imp/sec and a further increase is obtained at 8 imp/sec. There is a clear-cut elevation of the mean tension level during stimulation despite the fact that the amplitude of the individual contractions is less at 4 imp/sec than in the control periods.

In the in vitro experiments where no constant base-line was present and where spontaneous activity was more irregular the responses to nerve stimulation resembled in all respects those obtained in the in situ preparation

The responses obtained in the in vitro experiments are the result of activation of adrenergic vasomotor nerve fibres as judged from a pharmacological analysis similar to that presented for the in vivo experiments in Fig 4 The muscle response to nerve stimulation in vitro was thus abolished after exposing the preparation to guanethidine (1 mg/l in the bath) which did not interfere with the responsiveness to injected noradrenaline. Phenoxybenzamine blocked the excitatory responses to both noradrenaline and nerve stimulation. High concentrations of noradrenaline (1–10 mg/l) inhibited the spontaneous activity of the isolated rabbit portal vein after the alpha adrenergic blockade whereas nerve stimulation had no consistent effect The noradrenaline inhibition was abolished after propranolol (1 mg/l in the bath)

The tension responses of the in vitro preparation to nerve stimulation at graded frequencies were quantitated and expressed in per cent of the response to 16 imp/sec (Fig 7) Voltage and pulse duration were kept constant over the whole series of stimulations and were considered to be supramaximal when the response to a given frequency could not be further increased by changing stimulus strength. As in the in

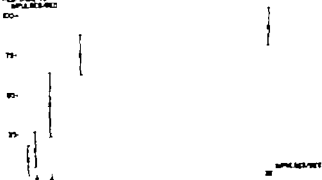


Fig 7 Diagrammatic representation of mean tension responses produced by postganglionic nerve stimulation in 10 preparations of rabbit portal vein in vitro. Responses expressed as per cent of response to 16 imp/sec. Mean values ± S D are indicated.

vivo experiments the tension response was maximated at 8 or 16 imp/sec. A comparison of the frequency-response curves of Fig. 5 and 7 shows a tendency to weaker responses in the lowest frequency range of Fig. 7.

A quantitative comparison between the tension responses to nerve stimulation and to graded concentrations of noradrenaline in the bath was made in four of the in vitro preparations. The threshold concentration of noradrenaline which elicited a tension response in the isolated rabbit portal vein was as low as 0.1 µg/l and with higher concentrations the log dose-response curve was linear up to 1 mg/l. The response to 10 mg/l was close to maximum. If this latter response to noradrenaline is considered as 100 per cent the maximal responses to nerve stimulation at 8 or 16 imp/sec range in the different experiments from 10 to 30 per cent. The maximal responses to nerve stimulation were approximately equivalent to those produced by 10—100 µg of noradrenaline per liter in the bath.

Discussion

The present report describes an innervated preparation of rhythmically active vascular smooth muscle from cat and rabbit portal vein, in which the tension responses to nerve stimulation could be studied in a quantitative way with the vessel in situ or isolated in the bath. In the abdominal veins there is a predominantly longitudinal orientation of the muscle cells (Kugelgen 1955) and the isometric tension recordings were done along this axis of the vessel.

One may speculate over the hemodynamic significance in vivo of the spontaneous rhythmic activity that characterizes this particular venous musculature in a number of species. The longitudinal phasic contractions can be associated with increases in the intravascular pressure as shown in experiments where the portal vein was closed off by ligatures and connected to a pressure transducer after being filled with Krebs solution. It is obvious that the rhythmic activity will be effective in the propulsion of blood through the vessel only on the condition that it is supplied with valves. According to Alexander (1963) there are numerous valvular structures within the mesenteric veins, but they are often rudimentary and incompetent to the extent that the intestinal vascular bed can be artificially perfused in the reverse direction for some minutes until edema develops. It is possible that the incompetent valves as well as the rhythmic activity of the mammalian splanchnic veins are reminiscences of mechanisms that are functionally more important in animals supplied with a portal heart as in *Myxine* (Fange, Bloom and Östlund 1963).

The longitudinal contractile responses of the portal vein will be used here as an experimental model for studying nervous control of rhythmic vascular smooth muscle without considering further the possible hemodynamic implications of the spontaneous activity in this particular vessel. The aim of the in vivo experiments was first to elucidate qualitatively the vasomotor supply to the portal vein by splanchnic and vagal nerve stimulation and to analyse the responses by pharmacological means.

Stimulation of the right and left splanchnic nerves both elicited tension responses in the portal vein which were due to active contraction. The fact that these responses

were abolished by local application of guanethidine indicates that they were due to stimulation of adrenergic nerve fibres which have been demonstrated histochemically in the portal musculature (Fuxe, personal communication). Splanchnic stimulation had virtually no effect on the rhythmicity of the vein after guanethidine or phenoxybenzamine which may justify the conclusion that adrenergic excitatory fibres are the only sympathetic fibres of importance for the control of the portal vein. This statement obviously rests on the assumption that the two drugs are specific in preventing transmitter release from adrenergic nerve endings and in blocking alpha-adrenergic receptors, respectively. Resistance vessels in the mesenteric circulation are considered to receive only constrictor fibres from the sympathetic outflow (Folkow 1955).

The question whether the vagal nerves contain efferent fibres innervating the vessels of the gastro-intestinal tract has been difficult to decide by experiments with vagal nerve stimulation and recording of regional blood flow due to the simultaneous changes in gut motility and secretion which will affect vascular tone indirectly. The metabolic factors that may influence the resistance vessels in such experiments should be less important for portal vein activity and it was considered of interest therefore to explore the effect of vagal nerve stimulation on the present in situ preparation. Slight and inconsistent increases in portal vein tension were the only effects obtained and they were not altered significantly by atropine, phenoxybenzamine or synstigmine. They may have been secondary hemodynamic phenomena due to the marked slowing of the heart and the increase in central venous pressure. Even if the results do not entirely exclude a vagal influence on the portal vein, partly because vagal pathways may have been damaged by the dissections, it appears safe to conclude that, if such an influence exists, it is insignificant compared to that of the splanchnic innervation. The results are thus consistent with the thesis that vagal nerves are not important for the direct neurogenic control of mesenteric circulation (Folkow 1955, Kewenter 1965).

The quantitative aspect of the sympathetic influence on portal vein tension was presented in the frequency response curves of Fig. 5 and 7. Changes in the level of mean tension produced by the different stimulation frequencies were expressed in per cent of the response to 16 imp/sec which was the maximal response obtained in the majority of the experiments. Frequency-response curves of similar configuration have been described previously with regard to sympathetic nervous influence on the nictitating membrane (Rosenblueth 1932), on resistance vessels of different vascular beds (e.g. Folkow 1952, Celander 1954), on consecutive sections of the vasculature (e.g. Mellander 1960), on the isolated pulmonary artery (Bevan 1962) and on several other autonomic effectors. These previous studies, as well as the present one demonstrate that the sympathetic nervous system exerts its full range of smooth muscle control at low impulse frequencies.

Despite this common feature of the frequency-response relationships described for the various effectors, there are certain interesting differences in the absolute magnitude of the responses and in the steepness of the frequency-response curves at low

stimulation rates. This may be exemplified by the far greater range of sympathetic control of cutaneous resistance vessels than of skeletal muscle vessels demonstrated by Celander and Folkow (1953) and the steeper frequency-response relationship obtained for capacitance vessels compared to resistance vessels in skeletal muscle (Mellander 1960). The present experiments with simultaneous recordings of portal vein tension, arterial and portal vein pressure and portal blood flow did not indicate any significant difference between the frequency response curves for the longitudinal muscle of the portal vein and for the smooth muscle of the resistance vessels when their responses, in terms of tension and flow resistance respectively, were expressed in per cent of the response to 16 imp/sec.

Very high concentrations of noradrenaline in the bath produced integrated tension responses of the isolated portal vein that were 4—10 times those produced by nerve stimulation at optimal frequency. This clearly indicates that the limit for the neurogenic response is not set by "saturation of all the receptor sites" or by inability of the muscle to contract further. Failure of the nerve fibres to conduct action potentials at faster rates does neither appear to be a likely explanation for the fact that the responses level out at 8 or 16 imp/sec. The absolute refractory period of mammalian C fibres is in the range of 4 to 6 msec only (Douglas and Ritchie 1962). It is generally held instead that it is the release of transmitter at the nerve endings that fails at high impulse frequencies (Folkow 1952, Brown and Gillespie 1957). It is notable that the excitatory junction potentials recorded in guinea-pig vas deferens on hypogastric nerve stimulation began to decrease in amplitude already at impulse rates as low as 3 per sec. (Holman 1964). Diffusion distances will be an additional factor limiting the neurogenic influence on vascular smooth muscle, where the nerve supply is usually arranged in a thin two-dimensional plexus outside the media (e.g. Fuxe and Sedvall 1964, Norberg and Hamberger 1964). In portal vein there is also a deep plexus (Fuxe, personal communication) but diffusion distances will still be considerable. The maximal contractile response to nerve stimulation might come closer to the maximal response to injected noradrenaline in muscles like that of the vas deferens where the dense three-dimensional plexus of terminal nerve fibres reduces the mean diffusion distances for the released transmitter. It is likely, on the other hand, that the intercellular conduction between the smooth muscle cells of the portal vein represents an important compensatory mechanism which greatly increases the effectiveness of the sparse innervation.

The changes in the pattern of muscle activity produced by nerve stimulation deserves some comment in this connection. An increase in the frequency of the major contraction waves of the portal vein at splanchnic nerve stimulation was indicated by the in vivo experiments but more clearly shown with postganglionic stimulation in the isolated preparation where rhythmicity was more regular (Fig. 6). A marked increase in contraction frequency was seen already at 4 imp/sec and this "positive chronotropic effect" contributed greatly to the elevated level of mean tension since the individual contractions were of lower amplitude than in the control period. It is not possible to determine from these recordings whether the increase in f

was due to stimulation of automaticity in the single pacemaker that appeared to operate in the control situation in Fig 6 or whether nerve stimulation induced ectopic activity The reduced contraction amplitudes at 4 imp/sec may signify a certain degree of desynchronization of muscle activity It is evident, at any rate, that a focal release of transmitter from a sparse supply of nerve terminals may increase considerably the mean level of contractile tension in a propagating vascular smooth muscle by an influence on pacemaker activity even if the transmitter itself does not reach the majority of the muscle cells to exert an inotropic action on them. A more detailed analysis of these phenomena in portal vein will require studies of both electrical and mechanical events associated with nervous activation. It is hoped that the nerve-muscle preparation described above will be a useful tool for further experiments on neuromuscular function in rhythmic vascular smooth muscle.

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Spread of Excitation in the Smooth Muscle of the Rat Portal Vein

By

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Abstract

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The longitudinal musculature of the rat portal vein is characterized by spontaneous, rhythmic contractions indicating effective intercellular conduction in this vascular smooth muscle. The present study was done in order to evaluate the relative importance of neurogenic and myogenic mechanisms for the spread of excitation in an isolated preparation of this vessel. A local anesthetic agent, in concentrations which blocked impulse transmission in autonomic nerves, failed to interfere with conduction in the smooth muscle of the portal vein whereas hyperosmolarity which is considered to preclude electrotonic spread of action potentials between muscle cells (Barr Dewey and Berger 1965) caused desynchronization of the contractile activity. It is concluded that conduction in this vascular smooth muscle is myogenic in nature. The fact that the portal vein, in contrast to most other vessels, is equipped with a double plexus of adrenergic nerve fibres (Fuxe, personal communication) does not seem to be important for coordination of its spontaneous contractions. The influence of the hyperosmotic environment on the electrical and mechanical activity of the portal vein is illustrated by experiments with sucrose-gap technique. The mode of action of hyperosmotic solutions is discussed.

Synchronization and co-ordination of motility in muscle organs require that excitation can spread over the contractile elements fast enough and in an ordered manner. The mechanisms responsible for this conduction in different kinds of smooth muscle have been studied and reviewed by Prosser (1962). It appears that the local nerve plexus plays an important role in this respect in certain types of invertebrate smooth muscle (e.g. Prosser and Melton 1954). A direct propagation of impulses between the individual muscle cells, myogenic conduction, is considered to operate in most visceral smooth muscles. The existence of true protoplasmatic bridges for this impulse transmission from cell to cell is not borne out by electron microscopy (Rhodin 1962) but Dewey and Barr (1962, 1964) have demonstrated local sites, the nexuses, where the membranes of adjacent cells make intimate contact with each other. These are considered to be the low-resistance bridges over which electrical conduction of impulses can take place (Barr 1963).

Vascular smooth muscle has been studied mainly in larger vessels and here it appears to be of the non-propagating type. This has led to the conclusion that vascular smooth muscle in general should belong to the "multi-unit" group as originally suggested by Bozler (1948). However, several vascular preparations, particularly from small vessels, do show functional characteristics which are typical of the propagating, "single-unit" type of smooth muscle i.e. spontaneous rhythmic contractions, responses to passive stretch etc. (see e.g. Bohr 1965, Johansson and Bohr 1966). Among the larger vessels such activity appears to be most consistent and particularly well synchronized in the longitudinal musculature of the portal and mesenteric veins where the phasic contractions have been shown to be associated with propagated action potentials (Cuthbert and Sutter 1964, Funaki and Bohr 1964, Axelsson *et al.* 1966).

The longitudinal muscle of these splanchnic veins forms a rather compact layer of smooth muscle cells (Sutter 1965) and intercellular contact may be intimate enough for myogenic conduction to take place (cf. Prosser, Burnstock and Kahn 1960). However, the portal vein has also been shown to receive a nerve supply which differs somewhat from that of most other vessels. Whereas arteries and veins in general are supplied merely by a thin two-dimensional plexus of adrenergic fibres on the outside of the media (e.g. Fuxe and Sedvall 1964, Norberg and Hamberger 1964) the portal vein has an additional network of adrenergic fibres located between the muscle layers (Fuxe, personal communication). It is conceivable that these nervous structures might contribute to the remarkable propagating properties of this vascular smooth muscle.

The present study is an attempt to evaluate the relative importance of neurogenic and myogenic conduction in the isolated rat portal vein by utilizing experimental procedures which may be assumed to interfere with each of these conduction mechanisms separately. A local anesthetic agent, in concentrations which were shown to block transmission in sympathetic nerves, was used to interfere with nervous mechanisms in the portal vein. Hyperosmolarity was used for blocking myogenic conduction. The rationale behind this was the finding by Barr, Dewey and Berger (1965) and by Barr, Dewey and Evans (1965) that the cellular contact at the nexuses were ruptured by exposing the muscles to a Krebs solution which had been made hyperosmotic by sucrose.

Methods

Experiments on sympathetic nerves. To be able to use local anesthetics and hyperosmotic solutions respectively for differentiating neurogenic and myogenic conduction in the portal vein it was considered necessary first to explore their effects on transmission in autonomic nerve fibres. This study was carried out on cats anesthetized with sodium pentobarbital, 30 mg/kg i.p. after induction with ether. The cervical sympathetic trunk was dissected over a distance of three to four cm. The proximal end was placed on a bipolar stimulating electrode and immersed with paraffine oil, whereas the remainder of the exposed nerve passed through a pool of physiological salt solution of the following composition in mmoles/l: NaCl 119; KCl 4.7; KH_2PO_4 1.18; $MgSO_4$ 1.17; $NaHCO_3$ 14.9; $CaCl_2$ 2.5; dextrose 5.5; sucrose 50; and calcium disodium versenate 0.026. This solution was bubbled with 96% O_2 and 4% CO_2 and its temperature kept at 37°C. It will be referred to as "normal solution" below. A Grass stimulator type 5C was used for nerve stimulation.

The quantitatively recorded mechanical responses of the nictitating membrane to sympathetic stimulation were used as an indicator of the intactness of impulse transmission in the nerve. The contractions of the membrane were recorded by an isometric tension transducer (Grass FT 03) operating an ink writing oscillograph (Grass polygraph). After repeated control responses had been established the physiological salt solution surrounding the nerve was changed to one with identical ionic composition but with an osmolarity increased to 690 mosmoles/l by the addition of sucrose. The responses to stimulation were followed for 15 min after which the normal solution was readministered to the nerve. After another series of control stimulations lidocaine (Xylocain, Astra) was added to a concentration of 0.3 mmole/l and its effect on the responses to nerve stimulation was studied.

In the first of these two experiments the nerve had been dissected proximal to the superior cervical ganglion; in the second experiment also the ganglion and a section of the postganglionic trunk were freed and exposed to the test solutions.

Experiments on synchronization of mechanical activity in the portal vein. This study was done on the portal vein of 10 rats weighing between 250 and 400 g. The animals were killed by a blow over the neck. The abdomen was opened and an approximately two cm long section of the vein was dissected out. The special muscle holder used for these experiments is illustrated in Fig. 1. A small side branch going into the preparation at its middle was anchored to a metal rod and the preparation was sunk into a small plastic well with an inner diameter of 5 mm. There were two openings on the sides of the well and these were covered by a thin rubber membrane. The two ends of the preparation, referred to below as the hepatic and the mesenteric end, were pulled out through holes in the membrane large enough to fit the diameter of the vein without pinching it. The holder was then lowered into a mantled organ bath filled with physiological salt solution of the above composition. Mechano-electrical transducers (Grass FT 03) were connected one to each end of the muscle preparation and a passive stretch of 200 to 500 dyn was applied. The angle at the central point of attachment was such that each of the two ends could be stretched and could contract independently without influencing mechanically the opposite end. Muscle tension as reflected in the electrical output of the transducers was recorded on the Grass polygraph.

An accommodation period of about an hour was allowed before the experiment was started. The interior of the plastic well was circulated with normal solution during this period. It was then possible, by exchanging the solution in the well, to expose the central portion of the vein selectively to the influence of the local anesthetic agent and to hyperosmolarity respectively. An interference with conduction in the vein by these procedures was expected to appear as a desynchronization of contractile activity in the two ends of the muscle which were both operating in the normal solution of the outer bath throughout the experiment.

Experiments on electrical activity of the portal vein. The sucrose gap method was applied to the study of electrical activity in smooth muscle by Burnstock and Straub (1958). A modification of this technique was utilized by Axelsson, Johansson and Jonsson (1966) and by Axelsson *et al.* (1966) for simultaneous recording of electrical and mechanical activity in the rat portal vein. The influence of hyperosmotic solutions on the electrical activity of this vessel is illustrated in the present study by experiments with this modified technique. A two cm long section of the portal vein and adjacent portion of the superior mesenteric vein was dissected out and mounted in the sucrose gap apparatus. In principle the mesenteric end of the preparation was depolarized by being superfused with physiological salt solution where NaCl was replaced by KCl. The central section of the vein (about 1 cm) went through the horizontal part of a narrow T-tube perfused with isotonic sucrose solution.

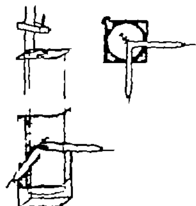


Fig. 1. Schematic drawing of muscle holder with plastic well utilized for selective exposure of the central part of the portal vein to the test solutions.

and the hepatic end was superfused with normal physiological salt solution or test solutions. The recording electrodes wer in contact with the solutions at the t ends of the preparation thus measuring the potential difference across the sucrose gap. Isometric contractions of the hepatic end of the muscle wer monitored by force-displacement transducer (Grass FT03) The signals from electrodes and transducer ere displayed on double beam oscilloscope and recorded continuously on moving film. For further details see Burnstock and Straub (1958) and Axelsson et al (1966)

Results

1 Effects of hyperosmolarity and lidocaine on impulse transmission in sympathetic nerv s

Fig 2 summarizes the effects on the contractile responses of the nictitating membrane of exposing the cervical sympathetic nerve to hyperosmolarity and to lidocaine. Stimulation of the proximal end of the nerve at 6 imp/sec 4 msec and 10 V elicited well reproducible responses as long as the nerve was in normal physiological salt solution. The mean of all responses obtained during the first and the second control periods have been set to 100 per cent in Fig 2. The relative changes in the amplitude of the contractions observed during the 15 min periods of exposure to hyperosmotic solution and to normal solution containing lidocaine are shown in this diagram. Stimulation characteristics were kept constant throughout the experiments. A small, non-significant decrease in the contractile response occurred as the preganglionic sympathetic trunk was exposed to hyperosmotic solution whereas no measurable change was seen in the experiment where both pre and postganglionic fibres were subjected to this procedure. Exposing the nerves to lidocaine led to a complete abolition of contractile responses within the 15 min of exposure It is concluded that lidocaine in a concentration of 0.3 mmol l is effective in blocking impulse transmission in autonomic nerve fibres and that hyperosmotic solutions fail to do this within the exposure periods used in this tudy

Effects of hyperosmolarity and lidocaine on conduction in at portal ein

The longitudinal musculature of the rat portal vein i haracterized by a spontaneous rhythmic activity consisting of phasic contractions correlated with bursts of spike potentials and separated by periods of rest Funak and Bohr 1964 Axelsson, Johansson and Jonsson 1966 Axelsson et al 1966) Simultaneous ecording of contractile activit in the hepati and mesenteric sections of the vein, as in the experiments reported below hows that exc tation spreads to initiate well synchronized contractions

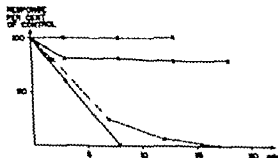


Fig 2 Changes in the esponse of the cat nictitating membrane sympathetic stimulation at 6 imp sec 4 msec 10 V obtained in one experiment her he preganglionic nerv ——— and in one her also he superior ervical ganglion and he postganglionic trunk ere exposed hyperosmotic physiological salt solution and to lidocaine ● l contrast to the local anesthetic agent, h perosmolarity does not interfere significantly ith nerv function.

The qua titatively recorded mechanical responses of the nictitating membrane to sympathetic stimulation were used as an indicator of the intactness of impulse transmission in the nerve. The contractions of the membrane were recorded by an isometric tension transducer (Grass FT 03) operating ink-writing oscillograph (Grass polygraph) After repeated control responses had been established th physiological salt solution surrounding the nerve was changed to one with identical ionic composition but with an osmolarity increased to 690 mosmoles/l by the addition of sucrose. The responses to stimulation were followed for 15 min after which the normal solution was readministered t the nerve. After another series of control stimulations lidocaine (Xylocain, Astra) was added to concentration of 0.3 mmole/l and its ffect on the responses to nerve stimulation was studied.

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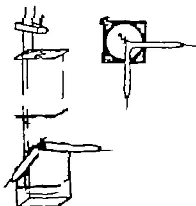


Fig. 1 Schematic drawing of muscle holder ith plastic well utilized for selectiv exposure of the central part of the portal ein to the test solutions.

Fig. 3 B shows the pattern of activity obtained five min after the normal solution in the well had been shifted to one with identical ionic composition but with a 100 per cent increase in osmolarity produced by adding 345 mmole sucrose extra per liter of normal solution. Exposing the centre of the muscle to this hyperosmotic solution is seen to dissociate the contractile activity in the two peripheral parts which operated in the normal solution of the outer bath. The mesenteric end shows major contractions with a frequency close to that observed during the control period (3A) whereas the hepatic end contracts at a slower rate. Apart from the larger contraction waves there are some smaller, less regular contractions on the mesenteric side. Hyperosmolarity thus seems to interfere with conduction in this smooth muscle so that it can no longer act as a "single unit". Fig. 3 B also shows that the two tension recordings were mechanically independent of each other in this type of experiment.

The effects of hyperosmolarity on synchronization are completely reversible as seen in the recording of Fig. 3 C obtained 10 min after return to normal solution in the well.

Doubling the osmolarity of the solution in the well by the addition of sucrose to the normal physiological salt solution caused a complete desynchronization of the major contraction waves in all experiments as it did in the example of Fig. 3 B. This effect was not produced consistently if osmolarity was increased by 75 per cent only and a 50 per cent increase did not in any case dissociate the contractile activity of the vein completely. It often happened, however, at these more moderate changes in osmolarity that one part of the vein began to lag behind the other with regard to the onset of contraction. One example of this is shown in Fig. 4. In the control situation (A) there was the usual simultaneity in the contractions of the two ends of the muscle and recording at higher paper speed (right) did not disclose any clear difference in their starting points. Exposing the central part of the muscle to a solution with a 50 per cent increase in osmolarity (B) failed to break up the contraction pattern as a 100 per cent increase did in Fig. 3 B but a time lag in the response

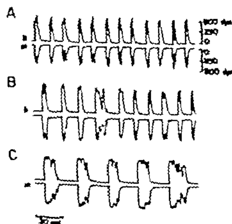


Fig. [illegible] Spontaneous contractions of hepatic and mesenteric end of the portal vein when the central portion of the preparation was exposed to normal solution (A), normal solution with lidocaine 0.3 mmole/l (B) and normal solution with lidocaine 0.6 mmole/l (C). Synchronization of activity was well maintained also in the presence of the local anaesthetic agent.

of the hepatic portion did occur. It seems justified to conclude that a slowing of conduction velocity in the centre of the muscle was partly or entirely responsible for this lag in the onset of tension development.

Fig. 5 shows the effect of lidocaine on the contraction pattern of the portal vein. A recording obtained 15 min after addition of lidocaine to the solution in the well at a concentration of 0.3 mmole/l (Fig. 5 B) does not differ from the control recording (A) with regard to contraction synchrony. It will be recalled that this concentration of the local anesthetic agent blocked nerve transmission effectively in the experiments reported under 1. Increasing the lidocaine concentration in the well to twice this concentration caused an increase in the duration of the phasic contractions but no dissociation of activity (5C). Still higher concentrations of the drug produced a strong excitatory response of the muscle so that an incomplete tetanic contraction occurred in both recordings. The results illustrated in Fig. 5, which are representative for the whole series of experiments, lead to the conclusion that local anesthesia fails to interfere with the synchronization of spontaneous portal vein contractions.

3. Experiments with the sucrose gap

Simultaneous recordings of electrical and mechanical activity with a sucrose gap method were done in two experiments on rat portal vein to illustrate the effects of high osmolarity. A more complete and quantitative analysis of osmotic influences on electrical and mechanical responses of vascular smooth muscle is carried out by Johansson and Jonsson (to be published).

Fig. 6 a shows the bursts of numerous spike potentials which are associated with the phasic contractions of the portal vein as previously described by Axelsson,

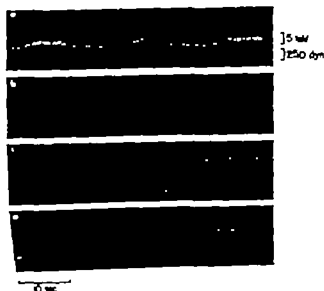


Fig. 6. Effect of hyperosmolarity and increased potassium ion concentration on electrical and mechanical activity of rat portal vein. Sucrose gap recording.
a Control in normal solution. b after one minute in solution with 690 mosmoles/l produced by adding sucrose to the normal solution. c Switching to hyperosmotic solution with potassium 59 mmoles/l (NaCl in solution from b substituted with equimolar amounts of KCl). d return to solution used in b.
Not complete inhibition of activity produced by hyperosmolarity and re-initiation of spike discharge with weak tension responses by potassium concentration which under iso-osmotic conditions would eliminate spike activity and cause contracture.

Johansson and Jonsson (1966) and by Axelsson *et al.* (1966). Shifting from normal solution to one with a 100 per cent increase in osmolarity produced by adding sucrose completely abolished spike discharge and phasic contractions within the first minute of exposure (Fig. 6 b). The level of the "resting potential" shifted slightly in the direction of depolarization, compared to the control in Fig. 6 a, but this small change may not be significant when obtained with the sucrose gap method.

It was shown in the experiments by Johansson and Jonsson, referred to above, that the effects on the portal vein of moderate increases in osmolarity could be counteracted or cancelled out by increasing the potassium ion concentration in the medium. Therefore it was decided to see in the present experiments whether potassium ions would be able to stimulate the vascular smooth muscle also when its spontaneous activity was completely inhibited by these high osmolarities. Fig. 6 c shows the result of switching from the hyperosmotic solution of Fig. 6 b to one with the same osmolarity but with a potassium ion concentration of 59 mmole/l i.e. 10 times that of the normal solution (NaCl reduced and substituted with equimolar amounts of KCl). This caused an immediate and clear-cut depolarization and a reappearance of spike potentials associated with weak phasic contractions. The tonic increase in the tension level seen in Fig. 6 c compared to a and b is probably the result of a gradual "shrinking" produced by the hyperosmotic environment. The regular spike activity beginning in Fig. 6 c persisted throughout the five min period of exposure to the hyperosmotic potassium-rich solution but terminated as the muscle repolarized on return to hyperosmotic solution with normal potassium ion concentration (6 d). A complete recovery to the control activity of Fig. 6 a was obtained with normal isotonic solution.

The results reported in Fig. 6 show that spontaneous electrical and mechanical activity in the portal vein is abolished by hyperosmotic physiological salt solution and that under these circumstances an increased potassium ion concentration can still evoke spike potentials with weak contractile responses. The second experiment which was done with the sucrose gap technique gave results which were the same in principle as those shown in Fig. 6.

Discussion

The present results show that the conduction mechanisms in the smooth muscle of the portal vein which are responsible for synchronization of its spontaneous contractile activity are not interfered with by a local anesthetic agent (lidocaine) in concentrations sufficient to block transmission in autonomic nerves. It seems as if the ability of this vessel to propagate and contract rhythmically cannot be ascribed to the particular arrangement of its adrenergic nerve supply demonstrated by Fuxe (personal communication) and referred to above. These nerve fibres influence the frequency and amplitude of the phasic contractions (Johansson and Ljung 1967) but they are apparently not essential for initiation or propagation of activity. This conclusion is further supported by the observations that adrenergic and cholinergic

blocking agents do not interfere with the spontaneous activity (Axelsson *et al* 1966, Johansson and Ljung 1967) It appears more likely therefore, that conduction in the portal vein should be myogenic in nature

The mechanisms involved in myogenic conduction in smooth muscles have been reviewed by Prosser (1962) and the different possibilities that he discussed may be considered with regard to the present experiments on portal vein. In the spindle muscle of *Golfingia* it was found that a response could not be conducted beyond a point of immobilization and it was concluded therefore, that conduction took place mechanically by pull of one fibre on another (Prosser Ralph and Steinberger 1959) Since synchronized activity was seen in the portal vein in the present experiments despite the fact that its central part was immobilized it is unlikely that conduction by stretch plays any important role in this vascular smooth muscle.

Specialized myo-muscular junctions" operating by release of pecific transmitters have not been demonstrated conclusively and most evidence speaks in favour of direct electrotonic spread of excitation from one cell to the other in many sceral smooth muscles (Prosser 1962, Barr 1963) Experiments by Roddie (1962) indicated that this mechanism of conduction is operating also in vascular smooth muscle from turtle arteries and veins. Dewey and Barr (1962, 1964) demonstrated the special contact sites, the nexuses, in myocardium and intestinal smooth muscle and suggested that they are the low-resistance bridges over which electrical conduction takes place This concept was supported by the further observations that hyperosmolarity interfered with propagation and that the nexuses were ruptured in electron micrographs from muscles treated with hypertonic sol tions Barr Dewey and Berger 1965 Barr Dewey and Evans 1965 In the light of these tudies one might assume that hyperosmolarity in the present experiment nterfered with synchronization in the portal vein by a similar mechanism and that a better development of nexal structures in this than in most other vascular preparations is responsible for its ability to propagate.

It was found, however that spike potentials could in fact be nitiated in the portal vein and recorded with the sucrose gap method even under hyperosmotic conditions by increasing the potassium ion concentration of the solution (fig. 6 c above If these spikes were conducted, which is most likely and not merely local phenomena they must mean that the mechanisms responsible for intercellular propagation were still functioning in the hyperosmotic en ironment The fact that these pikes gave rise to only feeble tension responses does not necessarily mean that they were not conducted over many cells for the weak contractions may just be due to the un oupling action of hyperosmolarity (Hodgkin and Horowicz 19 7 It is notabl that the potassium ion concentration used in the experiment of Fig 6 wa w ll above the concentrations which, under normotic conditions, are sufficient to liminat spike activity in the portal vein by producing a depolarization block Hyperr molarity was thus able partly to counteract the depolarizing action of an increased external potassium ion concentration. This reciprocal action on the portal em was found over a wide range of osmolarities and potassium concentrations by Johansson and

Johnson (to be published). A hyperpolarizing effect of hyperosmotic solutions was demonstrated with microelectrode technique in the guinea-pig taenia coli by Tomita (1966) and a similar mechanism may be responsible for the abolition of spontaneous activity in the portal vein (Fig. 6 b). That an increase in "resting potential" is not indicated by Fig. 6 b may be due to the fact that moderate changes in this parameter are not well reflected in sucrose gap recordings but it may also be worth mention in this connection that in the portal vein subnormal concentrations of potassium in the external medium seem to be associated with depolarization rather than hyperpolarization.

In view of these arguments and observations we must consider a reduced excitability by membrane "stabilization" as a mechanism by which hyperosmotic solution affects conduction through the central portion of the portal vein in the present experiments (Fig. 3 and 4). The relative importance of this factor in comparison with the possible decrease in nexal contact area cannot be determined from the present experiments. This question, which has been discussed for intestinal smooth muscle by Tomita (1966), does not, of course, influence our major conclusion that conduction in portal vein is myogenic rather than neurogenic.

Experiments like those shown in Fig. 3 and 4 might be expected to provide information also about the location of the pacemaker site in the venous preparation. When conduction block was produced in Fig. 3 the mesenteric end of the vein continued to contract at a rate similar to that seen prior to the block whereas the hepatic portion began to operate at a lower frequency. This gives the impression that automaticity was higher at the mesenteric end. The same conclusion may be drawn from the results of Fig. 4 where the mesenteric part contracts ahead of the hepatic one when conduction is slowed down enough to make a difference observable in the mechanical recording. Even if most of the experiments indicated that the spontaneous activity originated on the mesenteric side this was not consistently so. It is obvious that the result must be judged with great caution with regard to pacemaker site since automaticity in the different portions of the preparation may be greatly affected by the experimental conditions, particularly by the degree of passive stretch applied to the tissues. This question is currently subjected to further investigation.

Since neurogenic conduction does not seem to be important for coordination of activity in the portal vein it seems reasonable to assume that myogenic mechanisms predominate in this respect also in other mammalian vessels where the adrenergic nerve fibres are confined to the outermost layers of the media (e.g. Fuxe and Sedvall 1964, Norberg and Hamberger 1964). Such propagated phenomena as the ascending vasodilatation of skeletal muscle vessels (Hilton 1962) is, for instance, likely to be myogenic in nature. The phenomenon of vascular spasm may also be expected to spread by myogenic mechanisms.

The present study is largely based on experience and ideas obtained by one of us (B. J.) during a year at Dr David F. Bohr's laboratory, Department of Physiology, University of Michigan, Ann Arbor, USA. Dr Bohr's stimulating guidance in vascular smooth muscle research is gratefully acknowledged.

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From the Institute of Physiology, University of Oslo, Norway

Effects of Catecholamines on Pulmonary Blood Volume

By

A. Hauge, P. K. M. Lunde and B. A. Waaler

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Abstract

Hauge, A., P. K. M. Lunde and B. A. Waaler. *Effects of catecholamines on pulmonary blood volume.* Acta physiol. scand. 1967. 70. 323—333.

The effect of arterially injected adrenaline and noradrenaline on lung blood [illegible] has been examined in isolated, blood-perfused lung preparations from rabbit and cat. Both substances caused rapid and relatively marked reductions in the weight of the preparations, which was followed continuously. This weight reduction is interpreted as being due to constriction of capacitance vessels. This effect on the capacitance [illegible] was mediated via α-receptors, as addition of the α-inhibitor phentolamine abolished the response. Addition of the β-inhibitor propranolol did not interfere with the effect of the catecholamines on the capacitance [illegible]. Noradrenaline usually ga[illegible] an increase, adrenaline usually [illegible] decrease in pulmonary [illegible] vascular resistance in this preparation. The effect of the catecholamines on capacitance [illegible] was dissociated from the effects of these drugs on the [illegible] vascular resistance in the organ.

The pulmonary vascular bed was supposed to function as a blood depot already by Harvey (cit. 195[illegible]). In man, marked alterations [illegible] occur in pulmonary blood volume and neurohumoral mechanisms ma[illegible] be in[illegible]olved in these changes (Var[illegible]nauskas *et al.* 1963, Varnauskas 1966, Korsgren 1966).

Pulmonary [illegible] of d[illegible]fferent an[illegible]mal species ha[illegible] been [illegible] to react to nervous, humoral and gaseous stimuli by [illegible] p[illegible] of in[illegible]estigation (Daly 1958, Fishman 196[illegible], [illegible] 1965). [illegible] these 3 gro[illegible]ps of stimuli have all been analyzed mainly for their abilit[illegible] [illegible] change pulmonary [illegible] vascular resistance (PVR). Adrenergic stimuli ar[illegible] among these ha[illegible] [illegible] most markedl[illegible] affect PVR. Daly and his collaborators ha[illegible] [illegible] shown that the pulmonary [illegible] vascular bed in the dog is under the influence of adrenergic [illegible] [illegible] the [illegible] stimulation of which can increase PVR in isolated perfused l[illegible] [illegible] per[illegible] [illegible] (Daly 1958, Daly 1961, Allison, Daly and Waaler [illegible] [illegible] catecholamines will affect PVR in isolated lung preparations [illegible] [illegible] a summary of the subject Aviado (1965) concludes tha[illegible] the [illegible] noradrenal[illegible]ne in the lungs is a [illegible] vasoconstriction, while tha[illegible] [illegible] be [illegible]her a vasoconstriction or a vasodilatation.

Knowledge [illegible] pulmonary [illegible] vascular capacity may be influenced b[illegible] [illegible] stimuli [illegible] Information about the possible influence of ad

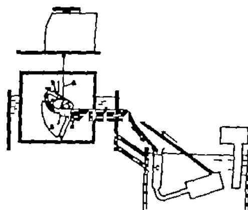


Fig 1 *Simplified diagram of the perfusion arrangement. (Recording arrangements and ventilatory system not included)*
A and E Communicating, thermostatically controlled waterbaths. B: Thermostat with water pump. C: Blood reservoir D: Head of perfusion pump. F Organ chamber G Lung preparation (somewhat enlarged) H Trachea with entilation cannula. I Perfusion inflow cannula (via right ventricle int pulmonary artery) with P sidebranch to manometer J: Outflow can ula (from left auricle) K Balance Q String from balance to tape around the heart ventricles. For further details see Fig 2.

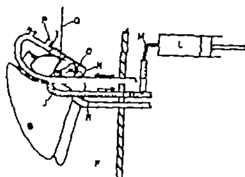


Fig 2 *Diagram with details of preparation and of arrangement for retrograde drug infusion into the left trium.*
L Infusion pump M Polyethylene catheter N Pulmonary artery O Left trium. Other symbols as in Fig 1

stimuli on capacitance vessels in the lung may be of special interest in view of the marked effect of such stimuli on PVR A few workers have noticed that adrenaline may reduce the total intrapulmonary blood volume in isolated perfused lungs (Gaddum and Holtz 1933 Daly 1957) We have examined further this effect of adrenaline and also of noradrenaline in isolated, perfused lungs of rabbits and cats. Both substances caused a marked reduction in pulmonary blood volume and this effect was dissociated from the concomitant one on vascular resistance A preliminary report on some of the data has been given elsewhere (Ha ge, Lunde and Waaler 1966c)

Methods

Some main features of the preparation and of the perfusion technique w ll be gi en below A more detailed description has been given lsewhere (H uge, Lunde nd Waaler 1966a

The lung preparations were taken from mature rabbits of both sexes and of bout 3 kg body weight The animal was anesthetized with an njection of pentobarbitone (Nembutal Abbott, 30—40 mg/kg) Heparin, 750 /kg of pure, powdered heparin (Nov) dissolved in saline as also injected intravenously before the chest was opened during positiv pressure entilation The caval veins were ligated, nd the preparation, consisting of both lungs with the lower part of the trachea, the heart and the pulmonary vessels, was taken out. The esophagus, the descending aorta nd most of the mediastinal connecti tissue was trimmed away from the preparation. A curved glass cannula for the perfusion inflow was inserted through hole in he wall of the righ entricle into the pulmonary artery (see Fig 1 and 2) The outflow cannula was tied int the left atri m (Fig 1 and 2)

In series of separate experiments the weight and blood content of the lungs proper as they were removed in the deflated condition from the bodies of 3 kg rabbits, were evaluated. The weights of 15 such pairs of lungs (without any additional tissue) ranged from 10 to 12 g. Blood volume in these deflated lungs was evaluated by thoroughly flushing through their vascular bed with plasma or with 0.9% saline. The hemoglobin concentration and/or the hematocrit was then measured in the effluent fluid. Between 4 and 5 g of blood were regularly found to be present in a pair of deflated lungs with this technique.

Perfusion technique. A simplified diagram of the preparation is given in Fig. 1. The pulmonary vascular bed was perfused at constant volume pulsatile inflow using Dale and Schuster pump with a frequency of 86 strokes/min. Within the pressure range used, the pump outflow was almost independent of the pressure against which the pump was working. The blood, which was circulated over and over again, was kept at 37—38°C by thermostatically controlled water baths surrounding the lungs, the blood reservoir and the pump head. In each experiment the blood flow through the lungs was measured by collecting the outflowing blood from the preparation in graded cylinder for a period of 10 sec.

Pulmonary arterial pressure was continuously recorded on kymograph drum with Marey tambour and was also followed with damped water manometer. The outflow tubing from the cannula in the left atrium (Fig. 1 and 2) was kept in fixed position and the pressure in the left atrium was thereby kept constant throughout an experiment. The left atrial pressure levels varied from one experiment to another between 2 and 5 cm of water.

Ventilation. Positive pressure ventilation with 5% CO_2 in air was employed. Peak inspiratory pulmonary pressure was kept at about 10 cm of water, expiratory pulmonary pressure at about 1.5 cm of water. The arrangement for the maintenance of these pressures and for recording the ventilation overflow was as described by Konzett and Rössler (1940).

Weighing technique. The preparation was placed inside plastic casing, where it was suspended by string tied around the heart ventricles (Fig. 1 and 2). (The heart was not perfused). The upper end of the suspending string was connected the base of Mettler balance, which combined suitable sensitivity with good damping of movement. This arrangement did not allow the direct measurement of lung weights. However, changes in the weight of the preparation could be followed with an accuracy of ± 0.05 g. Calibration of the weighing arrangement within the actual range of weight changes was carried out at the beginning and at the end of the experiment by placing suitable weight loads on the preparation.

Additional informations about the perfusate and treatment of the equipment used has been given by Hauge, Lunde and Waaler (1966a).

Drug addition. Drugs were injected into the pulmonary arterial tubing near the pulmonary artery in volumes of 0.5 ml of 0.9% NaCl solution.

Drug injections could also be carried out into the left atrium, by the arrangement illustrated in Fig. 2. The injection syringe which was driven by an apparatus for constant rat infusion (apparatus 1830,33—1 from B. Braun, Melsungen, W-Germany) was connected to thin polyethylene catheter. This catheter was led through the tubing for outflowing blood into the left atrium, where its tip was positioned near the openings of the pulmonary veins. After an experiment with such an injection into the left atrium, the position of the catheter tip was always controlled.

Adrenergic blocking agents were added to the blood reservoir in order to obtain rapid mixing with the perfusate.

List of drugs used

Adrenaline (Bitartrate d'adrenaline, Rhône Poulenc). Noradrenaline (1-noradrenaline d-bitartrate monohydrate, Philips-Roxane). Bradykinin (Synthetic Bradykinin, BRS 640, Sandoz A.G.). Adenosine triphosphate (ATP) Adenosine 5-triphosphate disodium salt, crystalline. From Equine Muscle, Sigma Chemical Company). Prostaglandin (PGE_1, kindly supplied by professor Sune Bergstrom, Department of Chemistry, Karolinska Institutet, Stockholm). Tri-cresol (distilled by Nyegaard & Co., Oslo, Norw.

Inhibitor

As inhibitor of α-effects of catecholamines: phentolamine methanesulphonate (Regitin, Ciba).
As inhibitor of β-effects of catecholamines: propranolol (Inderal®, I.C.I.).

Results

Perfusion was started 10—15 min after removal of the preparation from the animal. About 2 min later the lungs were inflated and their ventilation started. The onset of perfusion and particularly of ventilation led to a marked and immediate increase

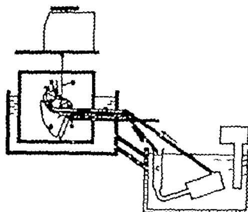


Fig. 1 Simplified diagram of the perfusion arrangement (Recording arrangements and ventilatory system not included)

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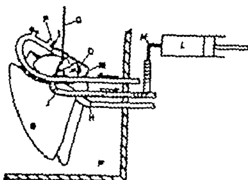


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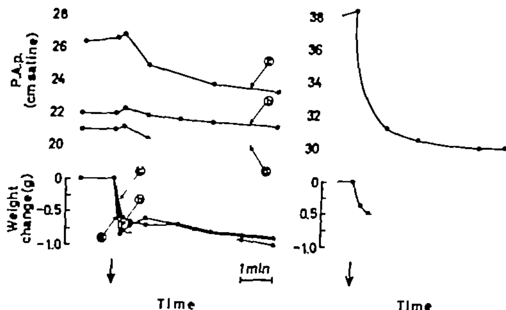


Fig. 3. Left part. *Effects on pulmonary arterial pressure (P.A.p.) and on preparation weight of 3 subsequent arterial injections of 25 µg of adrenaline on one and the same preparation.*
Isolated perfused lungs (I.P.L.) rabbit ♂ 3 kg. Perfusion at constant volume inflow. Blood flow through preparation (B.F.) 450 ml/min. Left atrial pressure (L.A.p.) 4.5 cm of water. Estimated pulmonary blood volume (P.B.V.) after start of ventilation 13 ml. Injections at arrow.
Right part. *Effects of injection into the pulmonary artery of 25 µg of adrenaline in another experiment.*
I.P.L., rabbit, ♂ 2.9 kg. B.F. 180 ml/min. L.A.p. 3.5 cm water. P.B.V. 11 ml.

the weight of the preparation. This increase in weight was interpreted as being due to an increase in the blood volume in the lungs — with their extrapulmonary vessels, and in the left atrium. The blood volume in the atrium was found to be of the order of 1—2 ml. Knowing the blood volume of the deflated, non perfused lungs (see *Methods*) and the increase in preparation weight upon perfusion and ventilation the total blood volume in the pulmonary vascular bed of the perfused preparations could be calculated to be from 11 to 13 g.

The total weight of a preparation did usually diminish slowly during the first 1—3 hr of perfusion, whereafter it remained almost stable for another 2 hrs. The weight reduction during the first part of the perfusion, which will not be discussed here, was of the order of 0.01—0.025 g per min. Most tests with catecholamines were carried out during periods with stable or almost stable weight of the preparation.

During blood perfusion of rabbit lungs a marked increase in PVR might develop, and this increase can be counteracted by tri-cresol (Hauge *et al.* 1966a). The effects of injected catecholamines were partly examined in preparations where no marked increase in PVR had yet occurred, and partly in preparations where the cresol had been added. The addition of tri-cresol (4 µg/ml of perfusate) did not influence the response of the preparations to catecholamines.

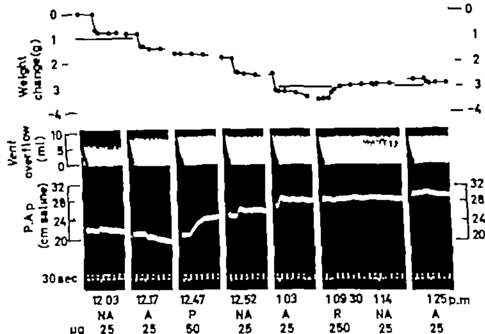


Fig. 4 *Effects on preparation weight and on P.A.p. of injections into the pulmonary artery of noradrenaline (NA) and of adrenaline (A) before and after the addition to the perfusate first of propranolol (P) and then of phentolamine (Regitin® (R))*

I.P.L., rabbit, ♂ 3 kg. B.F 230 ml/min. L.A p. 2 cm of water P.B.V 12 ml. Perfusion (constant volume inflow) started 11 23 a.m. Vent. overflow = ventilation overflow which equals effective stroke volume of respiration pump minus tidal volume (here about 40 ml) The decrease in tidal volume between 12.03 and 12.17 occurred spontaneously Abbreviations as in Fig 3

The lungs seemed to be well maintained during a 4 hr period of perfusion, which was the usual duration of an experiment. The reactivity of the vascular bed to various injected drugs did thus not decrease (Hauge *et al* 1966a, b) The tidal volume was gradually but slowly diminishing with time of perfusion, but it could be restored by reinflation of the lungs. There were no indications of oedema formation in the present experiments. The weight of each preparation was stable and showed no increasing tendency when perfusion was finished after about 4 hrs. Also the ratio of wet weight to dry weight (obtained by desiccation) was the same by the end of a perfusion, as in lungs that had just been removed from the animal.

The pH in the blood perfusate just after start of perfusion ranged from 7.35 to 7.45 with a mean around 7.4 A gradual fall in pH of some 0.02 units/hr was then usually seen during the perfusions, the preparations being continuously ventilated with 5 % CO_2 in air

Arterial injections of adrenaline in doses down to 2—5 µg usually caused a reduction in PVR and always a reduction in lung blood volume, as judged from a rapid fall in preparation weight These effects were maximal, or near maximal, with 25 µg of adrenaline (Fig. 3) and this dose was therefore used for most injections. Taking the actual blood flow to the preparation and the degree of mixing caused by the

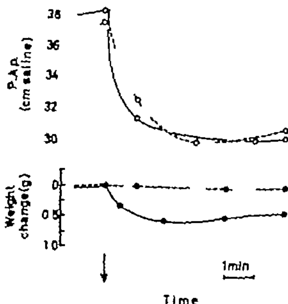


Fig 5. *Effects on P A.p. and on preparation weight of consecutive injections into the pulmonary artery first of prostaglandin (PGE₁) and then of adrenaline both in doses of 25 µg (at arrow)*

I.P.L., (constant volume inflow) rabbit, ♀ 2.9 kg B.F 180 ml/min. L.A.p 3.5 cm of water P.B.V. 19 ml. Broken lines: Effects of PGE_1. Continuous lines: Effects of adrenaline. Abbreviations as in Fig. 3.

pulsatile flow into consideration the adrenaline concentration in the blood entering the lungs after such an injection could be calculated to be about 1—2 µg/ml

The size of the reduction in PVR caused by 25 µg of adrenaline depended on the vascular resistance in the preparation prior to the drug administration If high before the injection the PVR could be reduced by as much as 40% by this dose of adrenaline. When a maximal dilatatory effect had been obtained, further injections of adrenaline sometimes resulted in a rise of PVR.

Noradrenaline in a dose of 25 µg usually gave an increase in PVR sometimes followed by a PVR reduction. Injections of this drug caused, however the same fall in preparation weight as did adrenaline (Fig 4)

Injections of adrenaline or noradrenaline did never cause any bronchomotor responses in this preparation (Fig 4)

The rapid weight reduction following adrenaline injections was a very constant phenomenon and was seen in all of 15 preparations tested. The weight reduction caused by a single dose of 25 µg of adrenaline corresponded to from 2 to 8 per cent of the total intravascular blood content and was usually of the order of 5—6 per cent of this content The effect on the weight was present whether adrenaline reduced increased or had no effect upon the PVR (Fig 3 and 4) In one experiment with cat lungs, injections of 25 µg of adrenaline caused an increase in PVR but a rapid weight reduction of similar magnitude to that seen in rabbit lungs.

In order to see if the left atrium was involved in the weight changes caused by injections of catecholamines infusions of adrenaline directly into the left atrium were carried out in 3 expts. The arrangement given in Fig 2 was used, and 0.25 ml containing 500 µg of adrenaline was infused in the course of 30 sec. No effects were

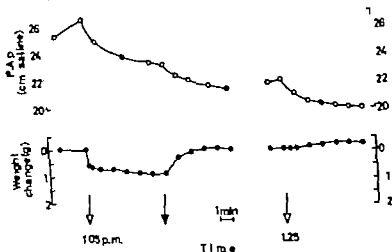


Fig. 6. Effects on P.A.p. and on preparation weight of adrenaline injections (25 µg at open arrows) into the pulmonary artery before and after addition to the perfusate of 500 µg of phentolamine (black arrow).
I.P.L., rabbit, ♂ 3 kg. B.F. 430 ml/min. L.A.p. 4.5 cm of water. P.B.V. 13 ml. Perfusion (constant volume inflow) started at 10.42 a.m. Abbreviations as in Fig. 3.

seen on PVR or on preparation weight until 30—45 sec after start of the atrial infusion, when a reduction in PVR as well as in lung weight occurred. This time interval corresponded to the total circulation time in the perfusion arrangement. Adrenaline had thus no capacity effect on the left atrium itself, but on vascular segments between the pulmonary artery and the left atrium.

The finding of adrenaline causing a reduction in PVR and at the same time a reduction in lung blood volume was rather surprising, and it prompted a comparison with another substance that reduces PVR in this preparation, namely synthetic prostaglandin (PGE_1). The effect of the two drugs on lung blood volume as evaluated by the weight changes in the preparation was very different. When doses of prostaglandin E_1 and adrenaline which were equipotent as regards the reduction in PVR were compared, adrenaline always caused the marked and rapid fall in preparation weight, whereas prostaglandin E_1 had either no effect on the weight or caused a very slight and gradual weight reduction only (Fig. 5).

For further analysis of the resistance and lung blood volume effects of adrenaline, adrenergic blocking agents were used. Adrenaline in doses of 25 µg was injected before and after the addition to the perfusate of 250 µg (or 500 µg) of the adrenergic α-receptor blocking agent phentolamine (Regitin®). The results are given in Fig. 4 and 6. The addition of this blocking agent resulted in a marked reduction or a complete elimination of the weight-reducing effect of adrenaline, whereas its PVR reducing effect was unaltered or slightly increased. Phentolamine had no effect of its own on preparation weight when added without a previous injection of catecholamine. The addition of phentolamine subsequent to an injection of adrenaline resulted, however, in an increase in the weight of the preparation. This increase in weight corresponded to the weight reduction caused by the previous injection of

adrenaline (Fig 4 and 6) Addition of phentolamine to the perfusate of the cat lung preparation also abolished the weight reducing effect of adrenaline. After such addition the PVR-increasing effect of adrenaline in the preparation was changed to a PVR-reducing one.

Addition to the perfusate of the rabbit lungs of the adrenergic β-blocking agent propranolol (Inderal®) in doses of 50 μg eliminated the PVR-reducing effect of adrenaline, whereas its effect on the weight of the preparation was unaltered. Addition of propranolol *per se* resulted in an increased PVR, but did not cause changes in the weight (Fig 4)

The effect of the α and β-blocking agents on the responses to noradrenaline injections were also examined. Phentolamine abolished both the PVR-increasing and the weight reducing effect of noradrenaline (Fig 4) Propranolol addition to the perfusate did not alter the weight response to noradrenaline injections, whereas the PVR-increasing effect became more marked after such addition.

The effect of catecholamines on preparation weight was usually of long duration, sometimes lasting 30 min or more. There was usually not a full spontaneous reversal of this effect, which lasted much longer than th decrease in PVR caused by adrenaline injections. It was possible to obtain a stepwise reduction of the preparation weight by a series of adrenaline injections. The effect of phentolamine when added during such a series of injections was a reversal of the weight reduction induced by the last dose of adrenaline only

Discussion

A decrease in lung weight can theoretically be due to either a reduction of lung blood volume or to a reduction in weight of extravascular tissue The weight reduction observed in connection with the injections of catecholamines is believed to ect a decrease in the intra ascular pulmonary blood volume This vascular response could not be secondary to airway changes, since bronchomotor reactions were not observed in the present preparation The onset of the weight reduction was very rapid, and most of the change took place within 15—20 sec after the injection. This rapidity of the response and also its reproducibility indicates that a change in vascular caliber is occurring When lung capillary pressure is altered, transcapillary flux of fluid with changes in the lung weight will be seen (Hauge *et al.* 1966d) Weight changes of this type are, however much more slow and gradual in their development Also a weight reduction caused by changes in transcapillary fluid balance would ha e to be linked with a reduction in lung capillary pressure It is difficult to believe that such a change would be common to the adrenaline and noradrenaline injections, with their different effects on total resistance. The fact that catecholamine-induced weight reduction was the same whether the PVR of the preparation increased or decreased thus also indicates that acti e reduction in the caliber of some capacitance vessels is taking place. The quantitative reversal of the adrenaline induced weight reduction by the addition of an α-blocker also supports this hypothesis.

The results thus indicate that catecholamines have a constricting effect on capacitance vessels in the pulmonary vascular bed. This effect was well reproducible and it was apparently dissociated from the effect of the catecholamines on vascular resistance, an effect which varied considerably. The capacitance vessels acted upon might contain a relatively large amount of blood, whereas their constriction seemed to have only a small effect on the total PVR.

The abolition by the α-blocker of the weight reduction induced by catecholamines indicates that the vasoconstriction involved is mediated via α-receptors. In the present preparation the effect of noradrenaline indicates that α-receptors are localized both in the resistance type and in the capacitance type of vessels. Such a dual localization of the α receptors is also indicated by the observations made when the α-blocker was added subsequent to an injection of adrenaline. The vascular resistance was then further reduced, whereas there was a concomitant increase in the pulmonary blood volume up to the pre adrenaline level (Fig. 6).

Injection of adrenaline caused simultaneous reductions in resistance and in vascular capacity. It is highly probable that also these two effects, the first one mediated through β-receptors, the other one through α receptors, are localized at different vascular sites. During constant volume inflow perfusion with constant left atrial pressure a reduction in PVR must be caused either by vasodilatation of open vascular channels or by opening up of new hitherto unperfused vessels. A reduction of blood volume can hardly be explained in other ways than as caused by a reduction of vessel caliber somewhere.

Our observations do not allow definit conclusions as to the localization in the pulmonary vascular bed of the resistance and capacitance vessels reacting to catecholamines. Earlier investigations by Hauge, Lunde and Waaler (1966a, b) suggest that in the lungs of rabbits the resistance vessels which react to other drugs must be situated mainly at the precapillary level, as an increase in pulmonary arterial pressure up to 50 mm Hg caused by for example eledoisin or ATP did not lead to pulmonary oedema.

The pulmonary blood volume changes caused by catecholamines might well to a large extent occur at the venous side of the capillaries. This would be in agreement with the situation in several parts of the systemic circulation in man (Eckstein and Hamilton 1957, Folkow and Mellander 1964, Wood 1965). In the findings of Eckstein and Hamilton (1957) adrenaline and noradrenaline infusions regularly caused large shifts of blood out of the forearm veins. This effect was inhibited by phentolamine.

The long duration of the capacity effect of the catecholamines, and the lack of a full spontaneous reversal of this response, cannot be fully explained. Nor do we know why added phentolamine which had no effect of its own on preparation weight, did reverse the weight response corresponding to the last given dose of catecholamine only. It is reason to believe, however, that these results may be related to the tendency in the preparation towards a slow spontaneous fall in the weight, which was seen during the first hr of perfusion. This development in the preparation, which

adrenaline (Fig 4 and 6) Addition of phentolamine to the perfusate of the cat lung preparation also abolished the weight reducing effect of adrenaline. After such addition the PVR increasing effect of adrenaline in the preparation was changed to a PVR-reducing one.

Addition to the perfusate of the rabbit lungs of the adrenergic β-blocking agent propranolol (Inderal®) in doses of 50 μg eliminated the PVR reducing effect of adrenaline, whereas its effect on the weight of the preparation was unaltered. Addition of propranolol *per se* resulted in an increased PVR, but did not cause changes in the weight (Fig 4)

The effect of the α and β-blocking agents on the responses to noradrenaline injections were also examined. Phentolamine abolished both the PVR-increasing and the weight-reducing effect of noradrenaline (Fig 4) Propranolol addition to the perfusate did not alter the weight response to noradrenaline injections, whereas the PVR increasing effect became more marked after such addition

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probably involve a slow progressive capacity reduction in the vascular bed might be enhanced by the catecholamines

The effects described on pulmonary blood volume seem to be rather specific for catecholamines. Several other vasoactive agents, which cause marked changes in PVR, have previously been analysed as regards their effects on preparation weight. Arterial injections of 5 μg of bradykinin (Hauge *et al.* 1966b) or of 25 μg of adenosine tri-phosphate (ATP) (Hauge *et al.* 1966a) did thus cause *marked increases in PVR* in this preparation. PVR increases induced by these substances, and of the same magnitude as those caused by 25 μg of noradrenaline, were not accompanied by any reduction in weight. During the very marked PVR increases caused by larger doses of these substances, the weight of the preparation was — if anything — increased. This finding has been interpreted as due to distension of blood vessels proximal to the constricted precapillary vascular segments (Hauge et al. 1966d)

Stimulation of sympathetic nerve fibres to the lungs has been demonstrated to cause *vasoconstriction both at a pre and at a post-capillary level in the pulmonary* vascular bed of the dog (Eliakim and Aviado 1961 Daly and Waaler 1961) In studies on the effects of stimulation of sympathetic nerve fibres to the lung the main emphasis has been more on changes in PVR, however than on possible capacity effects. With the marked effects of catecholamines on the vascular capacity of the pulmonary vessels in the rabbit in mind, it seems important to analyse more closely the effect of sympathetic nerve fibre stimulation on the vascular capacity of the organ. Such experiments are in progress in our laboratory

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Circulatory and Behavioural Effects on Electrical Stimulation of the Sympathetic Vasodilator Areas in the Hypothalamus and the Mesencephalon in Unanesthetized Dogs

By

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Abstract

BOLME, P., S. H. NGAI, B. UVNÄS and L. R. WALLENBERG. *Circulatory and behavioural effects on electrical stimulation of the sympathetic vasodilator areas in the hypothalamus and the mesencephalon in unanesthetized dogs.* Acta physiol. scand. 1967. 70. 334—346.

Blood pressure, heart rate, blood flow to the hind limb and behaviour were studied in conscious dogs during topical stimulation of the sympathetic vasodilator outflow areas in the hypothalamus and the mesencephalon. The responses obtained on stimulation were a rise in blood pressure, tachycardia and also an increase in hind limb blood flow which could be abolished by atropine. The effects on circulation caused by stimulation were essentially the same in the conscious and the anaesthetized dog. The behavioural changes on stimulation were relatively small and consisted of alterations in the degree of alertness. In many experiments marked circulatory responses were obtained on stimulation without any apparent behavioural reactions. Whether these findings indicate that the cholinergic vasodilator system acts more generally in circulatory homeostasis or is restricted to participation in specific situations as e.g. adjustments preparatory to muscle effort, is discussed.

Activation of the sympathetic vasodilator nerve outflow to the skeletal muscles in the cat and the dog by topical electrical stimulation in the hypothalamus and other supramedullary structures results in a characteristic cardiovascular reaction pattern. A pronounced increase in the skeletal muscle blood flow is produced (Eliasson *et al.* 1951, Eliasson, Lindgren and Uvnäs 1952) caused by an effect of the vasodilator nerves on precapillary resistance vessels (Folkow, Mellander and Öberg 1961, Renkin and Rosell 1962, Rosell and Uvnäs 1962). Vasodilatation in the skeletal muscles is apparently cholinergic in nature as it can be abolished by atropine. The muscular vasodilatation cannot be maintained in spite of continuous stimulation for more than about one min; then the blood flow gradually returns to prestimulatory values

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(Lindgren 1955 Folkow Mellander and Öberg 1961 Renkin and Rosell 1962) Stimulation of the vasodilator nerve outflow also produces vasoconstriction in the cutaneous and splanchnic vessels (Eliasson *et al.* 1951) as well as in the renal vessels (Feigl, Johansson and Löfving 1964) Cobbold *et al* (1964) showed that the vasoconstriction in the resistance vessels of the intestinal vascular bed is pronounced during the initial period of hypothalamic vasodilator outflow stimulation but after 1—2 min the blood flow returns to control values in spite of continuous stimulation.

Further electrical stimulation in the hypothalamus of the vasodilator nerve outflow produces release of catecholamines (Grant *et al* 1958) tachycardia and augmentation of cardiac contractile force (Rosén 1961) The net result of these cardiovascular responses is an increase in the cardiac output which is, at least initially distributed predominantly to the skeletal muscles.

Eliasson *et al* (1951) suggested, on the basis of their observations in anesthetized cats that activation of the vasodilator outflow might be part of a response to fright, rage and other situations requiring sudden muscular effort. Abrahams, Hilton and Zbrozyna (1960, 1964) held similar contentions and claimed that activation of the sympathetic vasodilator mechanisms prepares the animal for defence reactions. Except for the observations by Abrahams, Hilton and Zbrozyna (1960 1964) on conscious cats, our knowledge and consequent speculations about the physiological significance of the sympathetic vasodilator mechanisms are based on studies on anesthetized animals.

It could also be speculated that the vasodilator mechanisms might have a more general physiological significance in circulatory homeostasis than just being concerned with defence or other emergency reactions. This prompted us to undertake a series of studies on conscious dogs some of which will be described below The sympathetic vasodilator outflow was activated by topical electrical stimulation in the hypothalamus and the mesencephalon The cardiovascular response in terms of arterial pressure heart rate and blood flow to the hind limb was correlated with behavioural changes.

Methods

13 dogs weighing between 10 and 25 kg were used Under pentobarbital anesthesia (30 mg/kg, i. v.) and with aseptic technique the abdominal aorta was exposed through a mid-line laparotomy incision A polyethylene catheter (Clay Adams PE 205) was inserted into the aorta, directed cephalad for the measurement of arterial pressure No ligature was used to fix the catheter to the vessel The free end of the catheter was passed through the lumbar muscles and the subcutaneous tissue over the dorsum, emerging in the interscapular region The method is modified from that used in rats by Weeks and Jones 1960 For the measurement of arterial blood flow to the hind limb previously calibrated electromagnetic flowmeter probe of appropriate internal diameter was placed around the external iliac artery Leads from the probe were passed to the interscapular region and fixed to the skin A silk loop was put around the iliac artery distal to the probe emerging in the groin By pulling the thread it was possible to occlude the artery and thence to obtain zero flow readings whenever desired, a prerequisite for reliable quantitative values of the flow

At a second operation under chloralose anesthesia (100 mg/kg, i. v.) after an interval of at least ... days, stimulating electrodes were introduced into the hypothalamus and the mesencephalon. The head of the dog was placed in a stereotaxic instrument and up to 4 unipolar electrodes, 0.3 mm gauge stainless steel wires, insulated to one mm from the tip, were inserted through small burr holes in the skull The stereotaxic-coordinates used for producing vasodilator response were in the hypothalamus 18—23 mm rostral to the interaural plane, 1—3 mm lateral from the midsagittal

plane and in the mesencephalon 11—16 mm rostral to the interaural plane 2—3.5 mm lateral to the mid-line. With continuous electrical stimulation, each electrode was advanced downwards until there was an increase in the iliac blood flow. After testing with tropine (0.1—0.3 mg/kg, i.a.) which bolished the response the electrode was fixed in its position with acrylic cement nd connected to a microconnector anchored to the skull. The ground was provided by screw on the parietal bone.

After recovery period of 1—4 days, experiments were performed with the animal in the conscious stat. The dogs were held in quiet room standing i restraints. They became familiar with the recording equipment readily and did not seem to be disturbed except by unexpected noises. The arterial pressure was measured with Statham transducer (P23AA) connected to the aortic catheter. The blood flow was recorded with sine wave electromagnetic flowmeter[1]. The flowmeter probes were precalibrated i model experiments and only those with linear calibration curves were used. Calibrations were carried out under ideal conditions with minimal electrical resistance between the calibrating fluid and the sensing electrodes of the probe. It has been noticed, in experiments on cut dogs, that when the fit between the probe and the rtery is not perfect, the sensitivity decreases. This would mea that in some experiments the blood flow might be somewhat underestimated, fact that has no bearing on the interpretation of the results. A further description of the electromagnetic flowmeter will be published separately (Bolme and Carlsson, to be published). The heart rate was recorded with ordinate writer fed with either the pressure or the flow signals (Goldschmidt nd Lindgren 1962). All recordings were made on Grass model 5 Polygraph. Vascular resistance i the hind limb was derived from the mean blood pressure (MBP) and the blood flow (BF).

For electrical stimula ion of the hypothalamus or the mesencephalon, Grass model S4D stimulator was used with ts stimulus isolation unit. The stimuli consisted of square waves of 2 msec duration, 0.8—5 olts in intensity nd usually frequency of 80 cps.

Atropine sulphat (0.1—0.3 mg/kg) was also administered intraarterially through the aortic catheter to each animal in the conscious state t erify the nature of the vasodilatation elicited on central stimulation.

I three dogs flowmeter probes were also implanted around the superior mesenteric artery. The artery was exposed through subcostal ncision on the right side. Leads from the probes ere similarly threaded to the interscapular region. I these nimals blood flow was recorded both i the iliac nd the superior mesenteric arteries.

T recheck the position of electrodes in the vasodilator areas and the cholinergic nature of elicited response, terminal experiments nder chloralose anaesthesia were done on 12 of the dogs. In 2 of these animals the limb was skinned and ligature tied around the kle to compare the vasodilator response in the pure muscle preparation with that of the intact limb.

I all dogs the brains were perfused *in situ* with 10 formaldehyde solution. After ppropriat fixation the brains were grossly examined to erify the positions of the electrode ps.

Results

Observations on Conscious Dogs

a. *Circulatory responses.* In 15 dogs with 41 different electrodes, topical stimulation of the sympathetic vasodilator system in either the hypothalamus or the mesencephalon

[1] The flowmeter was designed by Allan Westersten and manufactured by AB Elema, Stockholm.

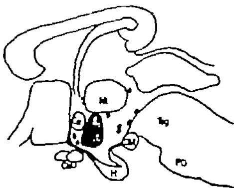


Fig. 1. Schematic drawing of sagittal section of part of dog brai. The stimulation areas are indica ed by shaded circles. The drawing includes points 1—3.5 mm lateral from the midsagittal plane. Ca. Commissura anterior. ChO = Chiasma opticum, CM = Corpus mamillare. MI = Massa intermedia, Teg = Tegmentum, H = Hypophysis, PO = Pons.

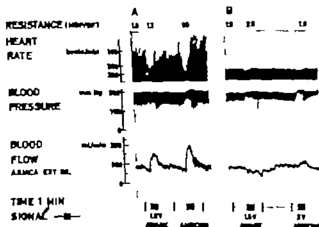


Fig. 2 Dog 10 kg. Effect of stimulation of vasodilator pathways in the hypothalamus before (A) and after (B) administration of atropine, 0.2 mg/kg i.

with threshold or supraliminal currents increased the external iliac blood flow within 2—3 sec. Fig 1 shows the areas stimulated by the electrodes. Atropine (0 1—0.3 mg/kg, i.a.) abolished or markedly reduced the vasodilatation following stimulation indicating the cholinergic nature of the elicited response. Along with the increase in blood flow tachycardia was always observed In the experiment illustrated in Fig 2 hypothalamic stimulation with 1.5 V increased the blood flow from 90 to 140 ml/min. The arterial pressure was practically unchanged and the calculated vascular resistance in the limb decreased from 1 8 t 1.2 The heart rate increased from 120 to 220 beats/min. Stimulation with 2 V resulted in a more pronounced response. The blood flow and heart rate were doubled and the arterial pressure rose. The calculated resistance decreased to 1 0 After atropine (0.2 mg/kg, i.a.) the vasodilator responses to repeated stimulations with 1.5 and 2 V were completely abolished In fact, stimulation after atropine produced small reduction of the blood flow probably as result of concomitant activation of constrictor fibres to the hind limb. In some of the other experiments an increase in arterial pressure was observed even with threshold stimulus intensity When the intensity was raised above the threshold level, elevation in arterial pressure occurred in almost all cases, indicating that vasoconstriction in non-muscular areas together with increased cardiac output had overcompensated the vasodilatation in the muscles

In 3 dogs the vasodilator response to central stimulation with various frequencies was studied. Stimulus frequencies ranging from 10 to 500 cps were effective (Fig 3) Maximal response was obtained within a range of 50 t 100 cps.

In 2 dogs the blood flow was recorded at the same occasion in the external iliac and the superior mesenteric arteries. Upon central stimulation of the vasodilator outflow a vasodilatation in the iliac vascular bed was accompanied by a vasoconstriction in the mesenteric area (Tabl 1) In one further dog the blood flow was recorded in the mesenteric artery alone Central stimulation was performed in the hypothalamic vasodilator area, as tested previously The vasoconstriction in the mesenteric

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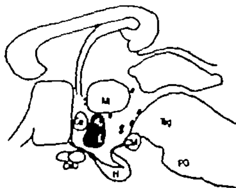


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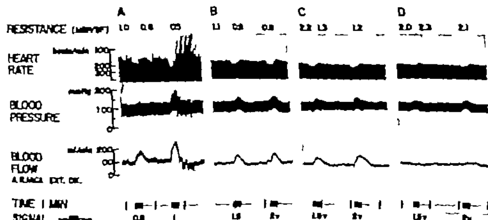


Fig. 4 Dog 15 kg Stimulation of vasodilator pathways in the mesencephalon.
A. Effect of stimulation with the dog conscious.
B. Effect of stimulation during chloralose anaesthesia (100 mg/kg i.v.).
C. The same as B after skinning of the right hind leg.
D. The same as C after administration of atropine 0.5 mg/kg i.a.

TABLE II. Behavioural response to hypothalamic and mesencephalic vasodilator outflow stimulation (37 electrodes in 14 dogs) T = Threshold stimulus intensity for producing muscular vasodilatation. For explanation of the behavioural response see text

Stimulus Intensity (V)	No response	Aware	Anxious	Excited	Rage
T	20	12	3	2	—
T+0.5	3	16	6	11	—
T+1	2	5	9	8	—
T+1.5	—	1	2	2	1

In order to test that the blood flow pattern in the intact limb essentially reflected the vasodilator response in the muscles, the leg was skinned in 2 dogs during the terminal experiment under chloralose anaesthesia. There was no qualitative change in the response to central stimulation after skinning (Fig. 4 B and C). In Fig. 4 B, with the hind limb intact, stimulation with 1.5 V increased the iliac blood flow from 95 to 150 ml/min and with [illegible] V to 160 ml/min. In 4 C, after the leg was skinned the resting blood flow was [illegible] ml/min. Stimulation with 1.5 V doubled the blood flow and with 2 V the increase was more pronounced.

b. *Behavioural response.* The behavioural responses to stimulation differed from animal to animal. Table II summarizes the reactions to increasing stimulus intensity. Stimulation with threshold intensity for producing vasodilatation in 20 out of 37 experiments failed to affect the behaviour to any significant degree. This can be seen in Fig. 3 where stimulation with the frequencies of 10, 200 and 500 cps did not give rise to any noticeable behavioural responses in spite of the circulatory changes consisting of tachycardia and vasodilatation in the hind limb.

As the first sign of behavioural reaction to stimulation in the vasodilator areas, the dog raised its head and looked around. This is signified by the term *aware*. When the stimulation intensity was increased somewhat more the dog became *anxious*. It would manifest a stepping movement and vigorously turn its head around, whine and might also urinate. Still further increase in intensity gave rise to *excitement*. The dog barked and attempted to escape, urinated as a rule, and occasionally defaecated. The pupils regularly dilated. When the stimulation intensity was increased even more, the reaction in most dogs was just a more pronounced excitement. However in 4 dogs, when the intensity was raised 2—4 V above the threshold intensity for producing vasodilatation, *rage* was evoked. With *rage* is meant that in addition to excitement, the dogs bared the teeth and tried to attack.

Atropine injected intra-arterially did not appear to affect the behaviour. Moreover the behavioural responses on stimulation were unchanged by atropine illustrated *e.g.* in Fig. 1 where the dog was aware of stimulation with 1 V and anxious with 1.5 V both before and after atropine.

Influence of Anaesthesia on the Circulatory Responses

In conscious dogs stimulation in most animals produced some increase in arterial pressure. During chloralose anaesthesia the pressor response was less pronounced. This is illustrated in Fig. 4, where A represents the stimulation when the dog was conscious. Stimulation with threshold intensity (0.8 V) gave rise to tachycardia and an increased blood flow. With 1 V the mean arterial pressure increased from 85 to 125 mm Hg. In B the dog was under chloralose anaesthesia. The heart rhythm was more stable and the stimulus threshold was elevated. On stimulation the blood flow and heart rate increased but the arterial pressure rose only slightly — from 105 115 mm Hg with 1.5 V and to 120 mm Hg with 2 V.

Further it was easier to make the dilatation persist by continuous stimulation of the vasodilator outflow during anaesthesia than during the conscious state. This may be due to the fact mentioned above, that the concomitant pressor response on stimulation during anaesthesia was abolished or less pronounced than during consciousness.

Influence of Central Depressants — Chlordiazepoxide and Morphine — on the Behavioural and Circulatory Responses

In 5 dogs chlordiazepoxide (Librium[R]) was given i.v. With a dose of 5 mg/kg the stimulus threshold for behavioural and pressor responses to stimulation was elevated while that for vasodilatation was unchanged. A representative experiment is shown in Fig. 5. Before the drug was given (A) stimulation elicited a typical response. 1.5 V caused tachycardia, a slight increase in the arterial pressure and a doubling of the blood flow. The calculated vascular resistance decreased from 0.58 to 0.31. The dog was alerted. With 2 V the vascular response was more pronounced and the dog now became excited during the stimulation. After the administration of chlordiazepoxide the dog became sedated but remained standing during the observa-

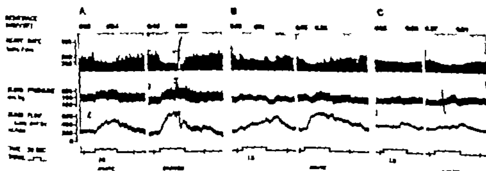


Fig. 5. Dog 18 5 kg. Stimulation of vasodilator pathways in the hypothalamus before (A) and after (B) the administration of chlordiazepoxide, 5 mg/kg i. Note that the pressor effect produced by the stimulation is influenced by the drug in contrast to the vasodilator effect which is essentially unchanged. At C is shown the effect of stimulation after atropine, 0.2 mg/kg i.a.

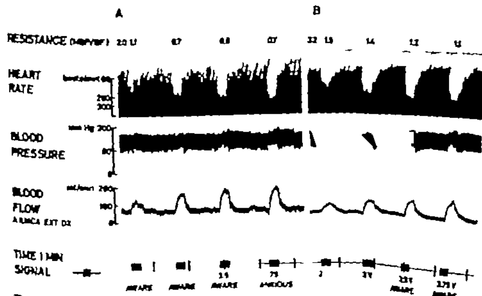


Fig 6 Dog 15 kg Effect of stimulation of vasodilator pathways in the hypothalamus before (A) and after B administration of morphine 1 mg kg i.m. ... the circulatory effect of stimulation ... the same before and after morphine ... behavioural response.

tion period — in the experiment for 45 min The arterial pressure and the blood flow were slightly reduced The ... remained almost the same. When stimulation was repeated with 1 5 V some tachycardia but no change in arterial pressure was produced Blood flow was more than doubled (from 200 to 450 ml min). There was no behavioural response With 2 V the tachycardia was pronounced, the arterial pressure remained unchanged and the blood flow increased about 3 times. The dog was alerted

The behaviour ... ponses could also be selectively blocked by morphine ... shows an experiment where the dog was sedated with 1 mg kg i.m. of

Before morphine (A) the threshold stimulation (2.5 V) increased the heart rate and blood flow with no change in arterial pressure. The dog became alerted. The resistance decreased almost 50 per cent from 2 0 to 1 1 The maximal circulatory response was obtained with 3 75 V with a marked tachycardia and a slight rise in arterial pressure. The blood flow increased 3 times from 60 to 180 ml/min and the resistance decreased to about 1/3 of the initial value from 2.0 to 0 7 The dog showed signs of anxiety After morphine the blood flow decreased from 60 to 40 ml/min (B) and the resistance increased from 2.0 to 3.2. The dog was sedated but did not sleep The stimulus threshold for circulatory responses was still 2.5 V which produced tachycardia, increase in blood flow but no change in arterial pressure. The resistance decreased about 50 per cent as before. The dog was not alerted either with 2.5 or 3 V With 3.5 V the dog became aware of the stimulation Stimulation with 3 75 V resulted in a marked tachycardia and an increase in arterial blood pressure. The blood flow increased about 3 times, from 40 to 130 ml/min As before morphine the resistance decreased to about 1/3 of the control value. The dog was aware of the stimulation.

Discussion

The technique of implanting an arterial catheter and electromagnetic flowmeter probes allows circulatory studies on conscious animals fo periods up to several weeks. However without reliable zero points, the blood flow recordings are only qualitative. Since most sine wave electromagnetic flowmeters in use lack a reliable electrical zero other reference values have been used In the present experiments zero points were obtained by total occlusion of the artery simply by pulling a string placed around the artery distal to the recordi g probe and thus allowing us to make quantitatively reliable recordings of the circulatory changes.

When study ng the functional significance of the sympathetic vasodilator system, interpretation of results requires the possibil ty to disti guish between a blood flow increase due to vasodilator nerve activity and that due to inhibition of vasoconstrictor activity and other haemodynamic changes. As pointed out under Methods we ha e identified the vasod lator response obtained on stimulation as cholinergic by using the specific blocking action of atropine

The cardiovascular responses observed on hypothalamic and mesencephalic electrical stimulation in conscious dogs were similar to those previously described for anaesthetized cats and dogs (Eliasson et al. 1951 Eliasson, Lindgren and Uvnäs 1952) The predominant findings were ncreased muscle blood flow accelerated heart rate and decreased mesenteric blood flow

The increase in arterial pressure on stimulation was a much more regular finding in the conscious than in the anaesthetized dog This pressor effect appeared to influence the vasodilator response to some extent When a pure vasodilator response without increase in arterial pressure was obtained the increase n blood flow could be maintained for at least 30—40 sec without changing the stimulus intensity

Attempts to maintain the dilatation by keeping the stimulation for a longer duration were unsuccessful especially in those experiments where there was a concomitant pressor response. This fact might indicate that vasoconstrictor fibres to the vessels of the muscles were activated together with the vasodilator fibres. This may thus reflect a certain antagonism between the adrenergic vasoconstrictor nerves and the cholinergic vasodilator nerves at the arteriolar level, as has been suggested by Folkow Öberg and Rubinstein (1964) They activated the vasodilator nerves centrally in cats and found that the vasodilator response could be almost abolished when the vasoconstrictor tone was elevated. Bolme, Ngai and Rosell (1967) have investigated the interaction between vasodilator nerve and vasoconstrictor nerve activities in dogs. A certain competition between the vasoconstrictor and the vasodilator systems was shown to exist both in conscious and in anaesthetized dogs. It was concluded, however that this competition, apparently much less than in cats, is presumably of minor physiological importance at least in dogs and that a sudden demand for increased muscle blood flow can be met by activation of cholinergic vasodilator nerves even during situations with increased vasoconstrictor tonus.

Under physiological conditions the cholinergic vasodilation could presumably be expected to overcome the prevailing vasoconstrictor activity at least initially This suggestion is supported by the fact that in many dogs with implanted electrodes, stimulation apparently activated both vasodilator and vasoconstrictor fibres simultaneously However the cholinergic dilatation always appeared immediately upon stimulation. On the other hand the vasodilatation gradually diminished after some 1 sec and became masked by the vasoconstriction. The physiological importance of the cholinergic vasodilator nerves may thus be restricted to the initial period of a request for increased muscle blood flow In order to maintain the dilatation for longer periods other mechanisms are certainly more effective as *e g* the accumulation of local metabolites following muscle work (Kjellmer 1965)

The observations by Orö, Wallenberg and Bolme (1965) might also indicate that the physiological role of the vasodilator system is restricted to short periods of sudden need for increased muscle blood flow and is not involved in the control of blood flow during long term exercise. When the hypothalamic vasodilator outflow in dogs was stimulated there was no sign of an increase in the plasma free fatty acid (FFA) level. On the other hand a significant rise in the FFA level was observed following stimulation in two areas in the brain stem not associated with sympathetic cholinergic vasodilatation but with increased blood pressure and heart rate. It is known from other studies that during exercise the skeletal muscles utilize FFA from adipose tissue as a source of energy (Carlson and Pernow 1959 1961 Basu, Passmore and Strong 1960 Friedberg *et al.* 1960 1963 Havel, Naimark and Borchgrevink 1963) Furthermore, the sympathetic nervous system plays a significant role in the mobilization of FFA from adipose tissue (Havel and Goldfien 1959 Rosell 1966) One may therefore speculate that if the function of the vasodilator nerves was to participate in long term exercise a release of FFA from adipose tissue should have paralleled the activation of the vasodilator system. In favour of the same assumption, that the

sympathetic vasodilator system does not interfere with the release of FFA are the findings by Ngai Rosell and Wallenberg (1966) that stimulation of the hypothalamic vasodilator outflow does not affect the circulation in subcutaneous adipose tissue.

It has been repeatedly suggested that the sympathetic vasodilator nerve outflow should function as part of the cardiovascular reaction pattern causing a redistribution of the blood flow to the skeletal muscles in anticipation of a major muscular effort. This reaction pattern has been discussed by Eliasson *et al.* (1951) as a response to emergency situations, as alarm reactions by Folkow Mellander and Öberg (1961) and as defence reactions by Abrahams, Hilton and Zbrozyna (1960 1964) These hypotheses about the functional significance of the sympathetic vasodilator outflow are based on circumstantial evidence In the cat vasodilator activation is observed to occur together with the above mentioned emotionally tinged reactions or at least by stimuli capable of eliciting such reactions provided a sufficient stimulus intensity is used. In our opinion these observations may justify the hypothesis that activation of the vasodilator nerves is part of a preparatory cardiovascular reflex in connection with defence reactions. However is the function of the vasodilator outflow restricted to such specific circumstances? The structure of the hypothalamus is complex and topical stimulation with electrodes is apt to activate various mechanisms which may or may not be functionally related. Conclusions as to the physiological significance of the various response patterns obtained have to be drawn with great caution.

In the conscious dogs emotionally tinged behaviour reactions were not characteristic nor constant features of the responses to stimulation of vasodilator areas in the brain stem. As described under Results, in the majority of experiments the predominant finding on vasodilator activation was either no behavioural response at all, or an "alerting" of the animal. The alertness could be enhanced to "excitement" by increasing the stimulus intensity In fact only 4 out of 15 dogs displayed "rage" during such stimulation, provided that the stimulus intensity was considerably raised above the "vasodilator threshold"

Abrahams, Hilton and Zbrozyna (1964) stated that in cats activation of the vasodilator nerves occurred concomitantly with the earliest signs of alerting The typical defence reaction did not develop until a more intense stimulation was given. This fact was taken to support the assumption that vasodilator activation was part of a preparatory cardiovascular reflex connected with defence reactions

The present observations on conscious dogs agree with those of Abrahams, Hilton and Zbrozyna (1960 1964) on conscious cats insofar that vasodilator activation occurs together with alerting reactions. They disagree from those of Abrahams Hilton and Zbrozyna in that defence reactions should be a concomitant response to hypothalamic vasodilator activation This species difference in the reaction pattern connected with vasodilator activation calls for cautiousness when drawing conclusions concerning the functional significance of the vasodilator outflow Its function might not be restricted to participation in defence reactions but embrace the nervous adjustment of the muscular blood flow in exercise at least in its preparatory phase. Perhaps the vasodilator nerves exert a tonic influence on the skeletal

muscle vessels, the vasodilator tone being related to the level of alertness of the CNS? These and other questions can only be solved by studies on conscious animals. Such studies on reflex activation of the vasodilator nerves are in progress.

Chlordiazepoxide reduces the pressor response obtained on electrical stimulation in the hypothalamus as demonstrated by Carroll *et al.* (1961) We have shown that with chlordiazepoxide, vasodilator responses to hypothalamic or mesencephalic stimulation remained unchanged while the behavioural response was bolished and the pressor and cardiac responses were considerably reduced. The same has been shown recently to be true also concerning the general anaesthetics halothane and cyclopropane (Ngai and Bolme 1966) Thus the vasodilator mechanisms seem to be more resistant to CNS depressants than the vasoconstrictor mechanisms.

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Acta physiol. scand. 1967 70 347—361

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Effects of "Diving" on Cardiac Output in Ducks

By

B. Folkow, N. J. Nilsson and L. R. Yonce

Received 9 February 1967

Abstract

Folkow B., N. J. Nilsson and L. R. Yonce. *Effects of "diving" on cardiac output in ducks.* Acta physiol. scand. 1967 *70* 347—361

Arterial and central venous pressures (AP and CVP) cardiac output (CO) and heart rate (HR) were recorded, and stroke volume (SV) computed, in awake ducks before during and after submersion, special attention being paid to the time course and extent of the induced changes. During rest CO was 1509 ml/min (S.D. 245) HR 244/min (S.D. 83) and SV 6.6 ml (S.D. 1.6) Immediately upon submersion CO and HR fell slightly and then showed a more gradual, up to twentyfold reduction within 1—2 min. SV decreased moderately despite a 10—20 mm Hg increase in CVP. During the immediate 1—2 sec postdive period HR increased to 300—500/min, with a sharp rise in AP while CVP fell. Within 10—15 sec a profound vasodilatation was established, AP and CVP returning towards normal. CO now reached 2500—4000 ml/min and SV increased beyond resting level. Thus, at the shift from submersion to emersion CO could increase up to fiftyfold within 10—15 sec. CO then returned towards control in 6—8 min. Results with vagal and sympathetic blocking agents suggest the following neurogenic events. During submersion inhibited sympathetic activity to the heart is coupled with a greatly intensified vagal discharge, reducing also ventricular contractility; the sympathetic vasoconstrictor fibre discharge is sharply increased. On emersion a complete reversal of this intense and differentiated pattern takes place within 1—2 sec. The receptors and central mechanisms involved are briefly discussed.

The physiological responses elicited in diving species upon submersion have several interrelated manifestations, which have attracted considerable interest since the classical studies of Paul Bert (1870). The most obvious and perhaps most widely studied responses are the bradycardia and the cessation of respiration. The intense bradycardia during submersion must either be accompanied by an unreasonable increase in stroke volume or by a peripheral vascular constriction, since mean arterial pressure is maintained or even increased in most diving animals.

Irving (1934) suggested that the bradycardia occurring during submersion forms part of a widespread and profound reflex adjustment of the cardiovascular system, so organized that the oxygen reserves present in the blood and the respiratory system are preferentially distributed to the essential tissues, the brain and the heart.

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Subsequent experimental data, though mostly indirect, strongly indicate that reflex vasoconstriction occurs in various vascular beds *other than the cerebral or coronary circulation*

In a recent review Andersen (1966) discusses these and *other aspects of the diving* response and he points to the paucity of data concerning the effect of submersion on cardiac output. Most data from earlier investigations have *in this respect been inferential*, suggesting that cardiac output must decrease since the arterial blood pressure can be maintained despite the intense bradycardia. *However Elsner et al.* (1964 1966) have recently measured the pulmonary arterial blood flow with an implanted ultrasonic flow meter in a sea lion. The cardiac *output was decreased* during spontaneous or command dives, but there were no figures to indicate the extent of the decrease. Elsner stated "Cardiac output varied in close accordance with the heart rate."

Shelton and Jones (1965) measuring stroke volume of diving frogs, found a decreased stroke volume as well as a decreased cardiac output during submersion. More recently Murdaugh *et al.* (1966) have measured cardiac output in diving seals (Phoca vitulina) by the Hamilton-Stewart dye dilution technique. They found that the cardiac output during the dive decreased to 6—36 per cent (mean 12 per cent) of the predive values, with no consistent changes in the stroke volume. The postdive values were not consistent though they suggested an increased cardiac output and stroke volume beyond the predive level.

The purpose of the present study was to determine in ducks the cardiac output and stroke volume during predive dive and postdive periods and to analyze the neurogenic mechanisms involved A preliminary report of the present and some related data has recently been published (Folkow Nilsson and Yonce 1966)

Methods

Experiments were performed on a total of 20 domestic ducks (Anas boscas 2.7—3.0 kg During the preparation period the duck was anesthetized i with [illegible] short acting barbiturate Narcotal (Astra 30 mg/kg The right brachial artery was cannulated with PE-160 and connected to P 23 V Statham *transducer for measuring arterial* blood pressure and heart rate When the central venous pressure was also to be measured, a cannula was inserted in the left brachial vein to a point near the right atrium (8—12 cm) In these experiments also the esophageal pressure was often *measured to give the true "transmural" pressure in the central vein.*

For determinations of the cardiac output the thermodilution and the cardiogreen methods were utilized. A thin cannula was inserted 7—10 cm into the right brachial vein with its tip placed close *to the right atrium The dye and the saline (0.2—0.3 ml)* at room temperature were injected simultaneously through the intravenous cannula, using the same syringe The dye was placed in the dead space between the syringe and the duck and the slug of saline was used to push the dye into *the duck.*

A sensitive thermistor placed 5—7 cm into the left brachial artery with its tip lying at the root of the aorta, was used for the measurement of the cardiac output by the thermodilution method *(Fegler 1954 Korner 1965* For the determination of the cardiac output by the cardiogreen method (for details see Nilsson 1963 arterial blood was withdrawn via a side tube of the arterial cannula used for the blood pressure recording This withdrawal, made with a syringe driven at a constant slow rate, pulled the blood past a photocell device for the spectrophotometric determination of the cardiogreen concentration in the arterial blood After this determination the small amount of

The authors want to express their appreciation to Dr P Korner and Civ Eng. L. Stage for their technical assistance in setting up the thermodilution technique for measuring cardiac output.

withdrawn blood was re-infused at the same rate. Repeated checks of the spectrophotometric unit indicated that the gradual desaturation of the arterial blood during the dive period did not per se change the baseline for the recording. — A small amount of heparin was given to avoid coagulation in the cannula and the syringe.

The arterial and central venous blood pressures and the thermodilution curves were recorded on a Grass polygraph, Model 5, while the cardiogreen dilution curves were recorded on a Kipp Micrograph recorder. These curves were later on extrapolated after plotting on semilogarithmic paper and the area under the curves was determined with a planimeter.

The skin areas surrounding the sites of cannulation were infiltrated with xylocaine for local anesthesia, and the duck was allowed to recover at least 30—60 min from the barbiturate anesthesia before the actual experiment was started. The animal was placed in a box designed to prevent escape but still allowing relatively free movements of the body and limbs. The head projected through a hole in the box at a natural angle and the duck was "dived" by immersion of the head in a small container of water usually for less than two minutes with at least 10 min rest before the subsequent dive. The animal was initially gently habituated to this procedure and usually tolerated the enforced head submersion with relatively little sign of agitation or stress. Sometimes the head was kept lowered in a steady position and "diving" was accomplished by raising the water level in the container.

Results

I. Time course of the cardiovascular responses to "diving"

A. Heart rate, arterial and venous pressures. The mean control heart rate of the ducks was 244/min (S.D. 83). The individual variations were thus large, and quite often the animal gradually increased the heart rate during the course of the experiment.

When the head of the duck was submerged in water the heart rate immediately decreased to 80—90 per cent of the control value. Continued submersion for 1—2 min usually caused a progressive bradycardia, down to values around 20 beats/min but values as low as 10—15 beats/min were not infrequent. In fact, after the ducks had been "habituated" to the enforced dive by repeated short submersions they often appeared more relaxed, even drinking and accepting food, and then the bradycardia during prolonged submersion tended to become more profound and regular. — Within 1—3 sec after cessation of the submersion the heart rate increased to values that were usually well over 300 beats/min, occasionally going as high as 500 beats/min.

Mean arterial blood pressure was between 100 and 175 mm Hg in all ducks during the control phase and generally changed very little during the diving period. The pulse pressure always increased somewhat during the dive because of a slightly decreased diastolic and, sometimes, a slightly increased systolic pressure. During the postdive period mean arterial blood pressure usually rose some 40—80 mm Hg within 2—4 sec, and then decreased gradually back to the control level in 20—60 sec.

The central venous pressure, which during resting conditions was usually close to zero, increased progressively during submersion up to 10—20 mm Hg when the diving response was intense, as judged from the extent of bradycardia. The time course of this often drastic increase in venous pressure was largely the same as the time course of the development of the bradycardia. Upon the interruption of the dive the raised venous pressure fell rapidly, usually reaching its normal value in about 10 sec. It was often checked by simultaneous recordings of the esophageal pressure

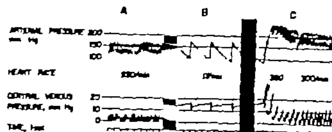


Fig. 1 A ke duck, 2.8 kg. Recordings of arterial pressure (AP) heart rate (HR) and central enous pressure (CVP) A. Predi control stat B Peak of the dive response after 50 seconds of submersion; note the intense bradycardia, the largely unchanged mean arterial pressure and the considerably raised entral venous pressure. C. The moment of emersion and the early postdi period; not the profound nd immediate rise in mean arterial pressure nd heart rat and the rapid fall in central enous pressure

that the given figures for increases in central venous pressure represented a truly transmural rise in pressure.

Fig 1 illustrates the characteristic changes in heart rate, arterial and central venous pressures in one duck during the "predive" control state (A) during the "peak" of the di e response after 50 sec of submersion (B) and at the moment of emersion and during the early postdive" period (C) In this particular duck heart rate decreased to about 17/min during submersion, at the same time as central venous pressure rose 13—14 mm Hg The development of these two changes followed each other closely both starting as a slight but almost immediate shift, which during the subsequent 40—60 sec became intensified more gradually — Note further the immediate onset of tachycardia and arterial pressure rise upon emersion and the fairly rapid return of central venous pressure towards the control level.

B *Cardiac output* Since the changes in cardiac output could be expected to be both extensive and rapid and therefore might involve difficulties in measurement, efforts were made to carefully standardize the procedures for measurements, to check possible artifacts and to compare the two methods used

For such purposes the cardiogreen and thermodilution methods were checked against each other in 41 determinations on five ducks during rest, the early dive and the postdive periods. The agreement was very good during rest and during the postdive period, the correlation coefficient, r being 0.85 (p 0.001 w th essentially all recordings falling well within the ± 20 per cent lines. The thermodilution method proved especially valuable during the postdi e period with its high flow values and rapid recirculation since this method is far less disturbed by ndicator recirculation than is the cardiogreen method

However there was an expected marked deviation of the values obtained by the cardiogreen method and the thermodilution method during the fully developed diving response. The sharply decreased flow through the cardiovascular system during this late phase of the dive must cause the i v injected temperature slug to equilibrate to a considerable extent with the tissue temperature during the slow passage through the heart and lungs. The area under the thermodilution curve will for such reasons be decidedly smaller than expected because of this "loss of indicator" Furthermore

there was also sometimes a slow drift in baseline during the head submersion. Therefore when the thermodilution curves obtained during prolonged dives could be used for calculations, they always gave *higher* values for cardiac output than the cardiogreen curves. The agreement was far better in early phases of the dive when the flow reduction was only moderate, but here, too, shifts in the central temperature of the duck could produce a disturbing drift of the baseline.

With the system used for measuring cardiac output, determinations with the cardiogreen method could be made only once during each of the dives" On the other hand, repeated measurements with the thermodilution method were performed in some experiments during the dive period, but this method tends to overestimate cardiac output more and more as it becomes reduced, as mentioned earlier However in the early dive period, where the cardiac output decrease was still moderate, the thermodilution method provided some general information about the time course of the decrease in cardiac output. Furthermore, to determine its time course more exactly during the dive and postdive periods, several dives were performed after suitable rest periods, and the cardiac output was determined with the cardiogreen method at different times during these repeated dives. The development of the bradycardia could be used as a check that the reflex cardiovascular adjustments were about equally well developed in the different dives. The cardiac output determinations from consecutive dives could then be plotted on the same time course to show the approximate relationship of various phases of the dive. As could be expected it was found from these measurements that the change in cardiac output had about the same time course as that of the heart rate change.

II Extent of change in cardiac output

Fig. 2 shows the relationship between cardiac output, heart rate and stroke volume before, during the "peak" of the dive and immediately after the dive in five ducks, which showed especially intense and well-reproducible diving responses to submersion During "resting" conditions the mean cardiac output of 49 determinations in these animals was 1509 (S.D 245) ml/min in the predive period, and the mean stroke volume was 6.6 (S D 1 6) ml/beat as corrected for a body weight of 2.7 kg. Stroke volume plotted against heart rate shown an inverse relation for all of the values collected during the predive, resting periods. Isocardiac output lines drawn on the graph indicate that the "resting" cardiac output remained relatively constant in spite of the large differences in heart rate.

When these five ducks were submerged for periods of 1—2 min (a total of 15 dives as shown in Fig 2) the cardiac output decreased to 66—500 ml min. The computed stroke volume decreased 1.0—4 1 ml/beat in 10 of the 15 dives it was not changed in one dive and was increased 1.0—4.2 ml/beat in 4 dives. On an average it decreased somewhat and it should be noted that this decrease in stroke volume occurred despite a consistent and mostly profound rise in central venous pressure.

Within the first 10—15 sec of the postdive period the cardiac output increased to values well above the predive cardiac output, reaching 2500—4000 ml/min or more.

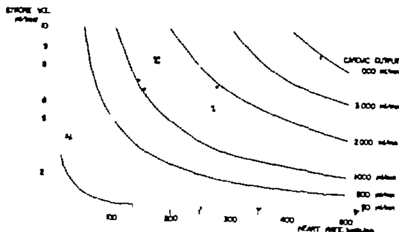


Fig. 2 Diagram illustrating the correlation between cardia output, heart rate and stroke volume during rest (filled circles) during the peak of the dive response (triangles) and during the early postdive period (crosses) in fiv ducks where measurements were performed in connection ith repeated periods of submersions. The isocardiac output lines are given, illustrating that "resting cardiac output is fairly constant, despit wide riations in heart rate All the figures for cardiac output and stroke olume are corrected for bodyweight of 2 7 kg

The stroke volume was now generally increased beyond the predive values, despite an often profound increase in heart rate and a central venous pressure that rapidly returned to the predive level

The low values for cardiac output and stroke olume d ring the dive period reflect the capacity of the cardiovascular system to adjust to submersion. However mean values for cardiac output and stroke volume were not calculated for the dive and the postdive periods, since the stress conditions of the laboratory procedure on e duck could not be well controlled. It was obvious that the intensity of the diving eflex was often markedly affected by the environmental situation The more relaxed d habituated" the ducks were to the situation, the more intense and regular was the diving response Therefore, the lowest alues for cardiac output and pulse rate during submersion should be the most relevant ones for the evaluation of the capacity of the diving reflex, since external disturbances were then probably min mal.

For such reasons the time course and extent of the d ving response of one duck which repeatedly displayed pronounced and regular bradycardia duri g the dive is shown in Fig 3 in some detail The mean heart rate cardiac output and stroke volume of three predive periods in this duck were *100 beats m n 1300 ml min and* 6.7 ml/beat, respectively Two dives of about 90 sec each were made and the cardiac output was determined in the final 20 sec. The heart rate during these dives decreased to 13—14 beats/min, the cardiac output decreased to 66 ml/min and the stroke volume to about 5 0 ml/beat. In the immediate 30 sec follow ng the emersion of the duck four measurements were made and the heart rate increased to a peak value of 390 be t /min, with a cardiac output of 3536 ml/min and a stroke volume of

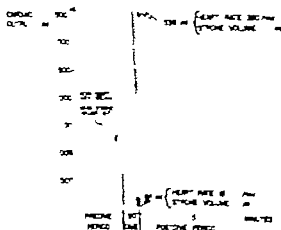


Fig. 3. Awake duck. 2.7 kg. Diagram illustrating the changes in heart rate, cardiac output and stroke volume during rest, during the peak of the dive response and after emersion in an animal, which in repeated dives regularly exhibited especially profound decreases in heart rate and cardiac output. Actual cardiac output measurements are given as crosses, the arbitrarily shaded area only serving to visualize better the sequence of events. Note the twentyfold decrease in cardiac output, and the reduced stroke volume, during submersion further the fiftyfold increase in cardiac output upon emersion, as compared with the dive, while the stroke volume is now increased beyond the predive level.

9.1 ml/beat. Values obtained 5—7 min after the submersion period indicate that the duck had then almost returned to the predive level.

III. *Effects of blocking sympathetic and vagal nerves to the heart*

Fig. 4 is the record of another duck showing the same general type of response, though in this case the bradycardia during submersion is not intense, only decreasing to 49 beats/min. This particular duck struggled and showed considerable signs of alarm while submerged and the bradycardia was interrupted by repeated brief periods of tachycardia, lasting four to five beats at a time. In this respect the duck shown in Fig. 4 differed considerably from that shown in Fig. 3. Cardiac output determinations were also made at varying intervals during the postdive period, as described above. These show the expected time course of the recovery of the duck on emersion.

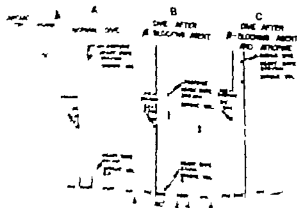


Fig. 4. Awake duck. 3.0 kg. Diagram illustrating in a similar way as in Fig. 3 the changes in heart rate, cardiac output and stroke volume before, during and after submersion. A. Normal dive. B. Dive after administration of β-blocking agent (Hassle 56/28, 1 mg/kg). Note especially the great reduced increase of cardiac output, heart rate and stroke volume in the early postdive phase as compared with A. C. Dive after administration also of atropine (1 mg/kg). Note especially how cardiac output now increases during the dive, as does also stroke volume, while heart rate remains constant. For details see text.

Following these control recordings, 1 mg/kg of a beta blocking agent (Hässle 56/28 Brändström *et al.* 1966) was slowly injected intravenously into the duck. Besides being a very potent beta blocker this drug exerts some beta stimulating effect of its own, so that its administration implies a slight but sustained beta receptor activation with respect to the myocardium. It will, on the other hand, entirely block the effects normally induced on the heart by its adrenergic nerve fibres.

Cardiac output, heart rate and blood pressure were now again determined during the predive, dive and postdive periods. *In the predive period heart rate is* down slightly and cardiac output is up slightly therefore, stroke volume has increased, though not to a significant extent. The consistent decrease in heart rate, seen in all ducks given this beta blocking agent, which exerts a slight beta stimulating effect of its own, supports the view that the sympathetic fibres to the heart of the "resting" duck exhibit a definite tonic activity

During the dive the reflex bradycardia is greater than before the beta blockade in this particular duck and the cardiac output is decreased more. This will be briefly discussed below Further the stroke volume is also in this case considerably decreased, suggesting that the negative inotropic effect on the heart — a fairly regular component in the diving reflex of the duck, especially considering the raised central venous pressure — cannot be ascribed only to an inhibited sympathetic discharge to the heart

During the postdive period after beta blockade, the cardiac output, as well as the heart rate, did not increase to the high values obtained before beta blockade in fact, it was sometimes of the same order of magnitude as the predive values (middle section of Fig 4) It appears, therefore that the sympathetic control of the heart is the primary factor respons ble for the sudden large increase in heart rate, cardiac output and stroke volume beyond the resting level occurring immediately after the emersion of the duck. A central inhibition of vagal tone during the immediate post dive period is present but is not by itself very effective in increasing the heart rate, cardiac output, or stroke volume beyond the predive level

Intravenous atropine (1 mg/kg) after beta blockade eliminated the typical diving response since heart rate now did not decrease at all and cardiac output actually increased during submersion (right section of Fig 4) The mean arterial blood pressure tends to increase after atropine presumably a consequence of the increase in cardiac output that occurs during submersion when both the vagal and sympthetic control of the heart is blocked

The further description of the experimental results calls for a brief comment concerning some of the results already shown in Fig 4 From this experiment it would appear as if the beta blockade *intensified* the reduction in heart rate and cardiac output during *submersion* However during the enforced submersion this duck showed clear signs of alarm a cond tion that was more or less unavoidable in the actual laboratory conditions. The possible influence of a superimposed state of alarm on the diving reflex is obviously of importance for the correct analysis of the primary mechanisms involved in the diving reflex.

It is well known that increased sympathetic activity to the heart and tachycardia form integral parts of the so-called defence reaction in most animals and also in ducks, and it was repeatedly observed how this response to alarm could greatly interfere and compete with the otherwise intense and regular bradycardia during submersion. In fact, such a competing influence was probably always more or less involved in the present experimental situation. If a beta blocker is administered in such a situation this would be expected to intensify the diving response, and the more so the more alarmed the duck was to start with. This is, in all likelihood, what happened in the experiment in Fig 4 and this experiment should therefore not *a priori* be taken to indicate that a reflex increase in sympathetic discharge to the heart forms a regular component of the normal diving reflex.

To explore this aspect further four ducks were exposed to repeated but brief submersions after a complete atropine blockade of their vagal heart fibres. Submersion still induced a reduction in heart rate, which was rather prompt in onset but slight in extent (10—15 per cent) especially when compared with that seen before the vagal block. A subsequent administration of the beta blocker eliminated this slight reduction in heart rate, induced by the submersion, suggesting that there is normally a component of inhibited sympathetic discharge to the heart in the diving response

Discussion

The resting cardiac output of the unanesthetized domestic duck was about 1500 ml/min under the present laboratory conditions. The weight of the heart was 15—21 g, a size comparable with the heart of the cat Yet, cats with the same body weight have, during superficial anaesthesia, a cardiac output of only 5—600 ml/min. In any case, it is apparent that ducks, with more than twice the cardiac output of cats for the same bodyweight, must have a very high flow of blood to some areas. In agreement with this it has been observed in ducks lightly anesthetized with nembutal, that „resting" muscle blood flow is usually very high, 3—4 times that in the cat when studied in similar experimental circumstances (Folkow Fuxe and Sonnenschein 1966) Also maximal blood flow capacity to the muscles is nearly 3 times higher in the duck.

The question arises whether this high cardiac output might represent at least partly a "luxuriant perfusion beyond the actual metabolic needs of the duck If so it would imply that the available oxygen reserve within the venous compartment is kept especially high. The large blood volume in diving species *per se* also implies a larger oxygen reserve available in the blood than in non-diving species. In another series of measurements of the oxygen content in arterial and mixed venous blood during rest in 5 ducks it was, however found that their A—V oxygen difference 3—5.5 ml/100 ml of blood, was the same or nearly the same, as in most other species (Folkow and Yonce unpublished) This means that the high resting cardiac output in the ducks, on the whole paralleled the metabolic demands, which are

high. Apart from this it might, of course, be possible that in diving species a brief phase of increased cardiac output, beyond the metabolic demands, sometimes precedes spontaneous diving. If so, this would be a fairly efficient way to charge the oxygen stores of the venous blood for the intended dive.

Whatever the case, the present results show that during a dive the cardiac output can be drastically reduced with a well maintained arterial blood pressure. In conjunction with our present understanding of the diving response, this can only mean that during the dive the brain is continuing to receive blood while the blood supply to most other tissues will greatly decrease or even stop. In general, the lower the cardiac output at a maintained arterial pressure, the better are the chances for the submerged animal to utilize the oxygen of the blood and air sacs for the essential organs, the brain and the heart. However it then actually calls for a complete or nearly complete obstruction of flow to most other tissues. Less intense reductions in flow will only mean an increased local extraction to the oxygen in the passing blood with no real saving of the total oxygen store, provided that this flow does not pass through shunts. Even with respect to the heart, which like the brain is also very sensitive to oxygen lack, some oxygen will be saved. The reason is that the intense bradycardia with correspondingly reduced cardiac output must sharply decrease the oxygen requirement of the myocardium. Some aspects of the distribution of the reduced cardiac output during diving were briefly outlined in a preliminary report (Folkow, Nilsson and Yonce 1966) and will be described in detail in a subsequent paper.

With respect to the time course of the cardiovascular changes upon submersion, the heart rate showed an immediate, though slight or moderate decrease, confirming earlier reports (Feigl and Folkow 1963; Andersen 1963). The by far largest decrease in heart rate developed gradually, however, over a period of 1—2 min with the most profound reduction occurring within the first 30—50 sec. The reduction of cardiac output showed a similar time course, as did also the rise in central venous pressure, while mean arterial pressure remained relatively constant throughout. These data indicate that the increase in the peripheral vascular resistance during the submersion period must be well correlated with the cardiac changes. The stable maintenance of the arterial blood pressure throughout the diving period suggests a control by some afferent modality. This strict balance between cardiac output and flow resistance does not seem to be only or even mainly dependent on the baroreceptors since a rapid withdrawal of arterial blood during a dive, sufficient to lower mean arterial pressure considerably, was found to leave the heart rate largely unchanged. The unavoidable unloading of the baroreceptors would, other things being equal, have caused a reflex increase in heart rate in this situation. Conversely a rapid injection of blood intra-arterially to raise the pressure did not produce any significant intensification of the bradycardia. The mechanisms, which during submersion normally balance the reduced cardiac output to the increased peripheral resistance changes in such a way as to maintain a relatively unchanged arterial pressure, are therefore not fully understood.

The decreased cardiac output during submersion was found to be the result mainly of the decreased heart rate, but this was usually combined with a decreased stroke volume. The effect of the decreased heart rate on cardiac output was anticipated, but the decreased stroke volume was surprising because of the often profound rise in filling pressure (10—20 mm Hg) during the dive. Aakhus and Johansen (1964) observed that the heart of the duck increased in size during a dive, a fact consistent with the present finding that central venous pressure increased during this period. Further in another study the same authors (Johansen and Aakhus 1963) also recorded an increase of central venous pressure during submersion.

A decrease in stroke volume, occurring when the filling pressure simultaneously becomes increased to the mentioned extent, suggests a considerable negative inotropic effect on the ventricular myocardium during diving, presumably of neurogenic origin where both the vagal and sympathetic components may be involved. The fact that the stroke volume decreased during diving also after administration of a beta blocking drug suggests that a vagal negative inotropic element affecting also the ventricles is, indeed, involved. This was substantiated in other experiments, which were briefly mentioned in a preliminary report (Folkow, Nilsson and Yonce 1966) and these results will be presented in a subsequent paper.

It may here be justified to attempt to integrate the present findings on the cardiac adjustments with other observations concerning the organization of the diving reflex. The complex and profound central nervous adjustments of the heart and peripheral blood vessels are evidently triggered by somatic and visceral afferents. Results by Feigl and Folkow (1963) and Andersen (1963) suggest that submersion of the nares initiates a reflex response which includes a slight but immediate bradycardia.

This immediate response, mediated at least predominantly by trigeminal afferents (Andersen 1963), seems to act as a "permissive" or facilitatory factor for the subsequent establishment of the fully developed diving response. This fully developed diving response is clearly dependent on the gradually developed hypercapnia and hypoxia, which must imply a stimulation of the chemoreceptors, and, perhaps, a direct stimulation of bulbar structures as well. Hypercapnia seems, if anything, to exert a stronger influence than hypoxia in this respect (Feigl and Folkow 1963, Andersen 1963). Results by Hollenberg and Uvnäs (1963) indicate that the chemoreceptors are indeed of crucial importance for the development of a typical diving response in the duck and this is suggested also by many earlier studies. It is further known from Daly and Scott (1958, 1962) studies in dogs that chemoreceptor activation results in a vasoconstriction in muscle, skin and intestine, and a decreased heart rate, if the simultaneously induced reflex increase in respiration is avoided. The same principal effects, though greatly enhanced and more rapidly initiated, are seen during a dive.

It is therefore most likely that the efferent discharge pattern involved in the phylogenetically ancient chemoreceptor reflex, forms the basic framework of the diving response, further that this efferent discharge is greatly re-inforced and even to

some extent anticipated by the more prompt activation of other types of receptors and nerve influences. As mentioned, *the primary influence seems to emanate from* the nares, and also other "somatic" receptor sites may be involved.

The so far mentioned influences are relayed at the bulbar level, since a strongly developed diving response can be obtained in decerebrate animals also (cf Andersen 1966) However it is also likely that more or less complex conditioned responses from higher brain centres are involved the autonomic adjustments taking place during submersion can, for instance, be mimicked by and re-inforced by topical stimulation in restricted hypothalamic mesencephalic structures (Folkow and Rubinstein 1964) Moreover it is known that *e g* seals can show prompt bradycardia even before their head becomes submerged (Scholander 1940)

The present results suggest that these different types of afferent inputs, needed for the full development of the diving reflex, lead to an efferent discharge pattern which is in principle organized in the following way The heart is exposed to a greatly intensified vagal activity affecting not only its rate but also its contractility (Folkow Nilsson and Yonce 1966, Folkow and Yonce 1967) while the prevailing sympathetic activity to the heart seems to become inhibited. On the other hand, the sympathetic discharge to the vascular bed is greatly increased with one noteworthy exception in form of the constrictor fibres to the cutaneous A—V anastomoses to be discussed in more detail in a subsequent paper (see also Folkow Nilsson and Yonce 1966) This means a profound differentiation of the sympathetic discharge with inhibition of the tonic activity to the heart and intensification of the tonic activity to the blood vessels, with exception of the cutaneous A—V shunts

Immediately after the dive this characteristic reflex discharge pattern changes into its absolute reverse There is a practically complete inhibition of the vagal activity combined with a greatly increased sympathetic discharge to the heart and, at the same time the sympathetic discharge to the vascular bed appears to become virtually abolished throughout all accomplished within a few seconds. The abolishment of the vagal influence on heart rate occurs almost momentarily and, since central venous pressure is still high this will lead to a prompt increase in cardiac output However as judged from observations in other species, it may be expected that the shift from intense vasoconstriction to full vasodilatation is not quite as rapid taking perhaps 10 sec, even if the cessation of constrictor fibre discharge is immediate This slightly delayed peripheral vasodilatation probably explains the temporary often considerable rise in arterial pressure in the immediate postdive period (Fig 1)

In general the mentioned opposite changes in activity of the sympathetic nerve supply to the heart and blood vessels are contrary to the old concept that the sympathetic activity should always be more or less generalized They represent one of several examples to indicate that the sympathetic system is designed for highly differentiated action which, when needed as in the diving response allows for increased discharge to one area and decreased discharge to another

The receptors and afferent pathways involved in this remarkable and prompt reversal of the efferent discharge pattern are not fully known However afferent

impulses from pulmonary stretch receptors and other parts of the respiratory system, initiated when respiration again starts after the dive, seem to be involved (e.g. Folkow and Rubinstein 1964). Further, as soon as the blood leaving the lungs is again oxygenated and reduced in its carbon dioxide content, the chemoreceptor drive is first rapidly reduced and subsequently decreases more gradually along with the normalization of blood pH as the lactic acid in the blood is eliminated.

The power of these specific nervous discharge patterns to adjust the cardiovascular system during and after a dive are impressive even in such a relatively poor diver as the duck. In some of the present experiments cardiac output decreased some 20 times below the resting control value within 1—2 min of submersion. A few seconds after cessation of the dive cardiac output was found to increase up to 50 times above the value recorded during the dive, reaching figures that were 2—3 times above the values during resting conditions.

Interference with any of the neurogenic links involved in these dramatic cardiovascular adjustments will grossly derange or even largely abolish the characteristic events during and immediately after a dive. Thus, when atropine or vagal denervation eliminates the decrease in cardiac output during the dive, the balance of the baroreceptor reflexes seems to prevent the intense vasoconstriction, and the animal will rapidly drown if kept submerged. Likewise, a beta blockade eliminated to a great extent the tachycardia and the cardiac output increase during the postdive period, which is probably of great importance for the rapid restitution of the metabolic equilibrium.

These blocking drugs also help to shed light on the neurogenic mechanisms involved. Thus, it becomes clear from the experiments where a beta blocker was used that a central inhibition of the vagal effect on the heart normally supplements the sympathetic excitation of the heart in the postdive period since there was still some increase in heart rate in this period after beta blockade. Similarly, the observation that a slight but clear reduction in heart rate took place upon submersion after a vagal block, but vanished after addition of a beta blocker, suggests that during the dive a sympathetic inhibition to the heart is normally associated with the clearly evident vagal activation. The two nervous links to the heart seem to act in a reciprocal fashion in both the dive and postdive periods, but in reversed directions in these two phases.

A duck in a diving state thus adjusts to the anoxic environment by conserving the oxygen stores for the most important tissues for survival. On the other hand, a duck in the alarmed state will usually attempt to escape by flying away. The cardiovascular adjustments, in form of the "defence reaction" which are required and known to be elicited in the alarmed state, are, in practically all respects, opposite to those of the diving state and can be elicited also in the duck (Feigl and Folkow 1963). This defence reaction is characterized by tachycardia, an increased cardiac output and a decreased peripheral resistance in the skeletal muscles, at least in the early phases of the reaction. The physiological balance of the autonomic control of the heart and blood vessels will naturally become affected in situations where these oppo-

some extent anticipated by the more prompt activation of other types of receptors and nerve influences. As mentioned, the primary influence seems to emanate from the nares and also other 'somatic' receptor sites may be involved.

The so far mentioned influences are relayed at the bulbar level since a strongly developed diving response can be obtained in decerebrate animals also (cf. Andersen 1966). However it is also likely that more or less complex conditioned responses from higher brain centres are involved: the autonomic adjustments taking place during submersion can, for instance, be mimicked by and re-inforced by topical stimulation in restricted hypothalamic mesencephalic structures (Folkow and Rubinstein 1964). Moreover it is known that e.g. seals can show prompt bradycardia even before their head becomes submerged (Scholander 1940).

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In general, the mentioned opposite changes in activity of the sympathetic nerve supply to the heart and blood vessels are contrary to the old concept that the sympathetic activity should always be more or less generalized. They represent one of several examples to indicate that the sympathetic system is designed for highly differentiated action which, when needed as in the diving response, allows for increased discharge to one area and decreased discharge to another.

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directed responses are simultaneously induced. As already described in "Results" it was frequently observed how the ducks, when alarmed, showed irregularities in their diving responses in form of intermittent periods of tachycardia, brief pressure rises, etc. suggesting an intermittent breakthrough of a competing "defence reaction". This evidently tended to again enhance the reduced cardiac output, as suggested by the actual measurements in such situations and also by the much accelerated "run off" in the systolic pressure pulse whenever such a breakthrough in heart rate occurred. The "calmer" and more relaxed the ducks were, the more intense and regular was usually the diving response.

The situation may, however, be very different in diving animals where alarm situations normally lead to diving as the natural way of escape. Here it may be expected that the autonomic adjustments typical of the diving reflex, rather than those characterizing the above mentioned "defence reaction", are preferentially induced when such animals become alarmed. This view is supported by the observation that sudden sounds, threatening movements, etc., usually elicit a prompt bradycardia in seals (Scholander 1942) rather than the tachycardia typical of the "defence reaction" seen in e.g. ducks when similarly exposed. Therefore, while an alarm situation in the duck tends to interfere with and suppress the diving reflex, as was often seen in the present experiments, it might re-inforce the diving reflex in species like the seal. This might be the explanation of the finding by Elsner *et al.* (1966) that cardiac slowing is much more profound in sea lions and harbour seals when forced to submerge than when trained to dive on command.

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In preliminary experiments various combinations of ankle and knee muscles were examined. Some inhibitory effects were often found but they were usually inconsistent and weak. However the gastrocnemius-soleus was regularly found to produce effects on DSCT units monosynaptically activated from primary endings (Ia DSCT units) of the tibialis anterior extensor digitorum longus and the flexor digitorum longus muscles. The present report concerns some qualitative observations on the inhibitory interaction between these two pairs of ankle muscles.

Methods

The experiments were performed on nembutal anesthetized cats. One hind leg was denervated except for the muscles under study. These were the gastrocnemius-soleus (GS), the flexor digitorum longus (FDL) and the tibialis anterior—extensor digitorum longus (TA-EDL). The FDL and TA-EDL tendons could be loaded with weights. The TA- EDL tendons were tied together so that these two muscles were always stretched simultaneously. The ipsilateral ventral roots LVI, LVII and SI were always cut. The activity of single axons was picked up by micropipettes in the ipsilateral dorsolateral funicle and recorded on moving film. Full details about the methods have been given in preceding paper (Jansen *et al.* 1966)

The experimental procedure was to identify Ia DSCT units (as described in Jansen *et al.* 1966) from either of the innervated muscles and to examine the possible effects of stretch or contraction of any of the other muscles. Inhibitory effects were then recognized as reduction in the firing frequency of the unit.

Results

GS effects on TA EDL units. Contrary to our expectations passive stretch of the gastrocnemius soleus muscle had rather little effect on the firing frequency of TA EDL Ia units. Altogether 17 such units have been examined during stretch of the triceps muscle. For 10 of them no reduction in firing frequency was noticeable during triceps stretch. The other seven units showed a weak to moderate reduction in firing fre

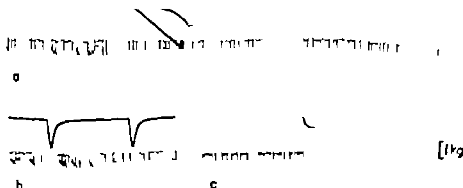


Fig. 1. Effect of GS stretch and contraction on TA-EDL unit. a, Top trace, GS tension. Second trace GS length. Final length about 4 mm less than full extension of GS [initial length 14 mm shorter]. Third trace action potentials of TA EDL unit. Insignificant effect of GS stretch. b, Same unit. Top trace GS tension during twitches, elicited by submaximal single shocks to muscle nerve. c, Same unit. Top trace GS tension during tetanic contraction elicited by tetanic [illegible] (100 sec) stimulation of SI ventral root. Load on TA EDL 100 g and time mark [illegible] sec for all records. Tension bar applies to all records.

Acta physiol. scand. 1967 70 362—368

From the Institute of Neurophysiology and Institute of Physiology University of Oslo, Norway

On the Inhibition of Transmission to the Dorsal Spinocerebellar Tract by Stretch of Various Ankle Muscles of the Cat

By

J K. S Jansen K. Nicolaysen and L. Walløe

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Abstract

Jansen J K. S K. Nicolaysen and L. Walløe, *On the inhibition of transmission to the DSCT by stretch of various ankle muscles of the cat* Acta physiol scand 1967 70 362—368

Dorsal spinocerebellar tract neurones activated from primary endings of muscle spindles (Ia DSCT units) are adequately excited from one muscle or closely synergistic group of muscles. Inhibitory effects by stretch or contraction of other muscles have been examined in anaesthetized cats. Consistent inhibitory effects were obtained from the gastrocnemius-soleus (GS) muscle on Ia DSCT units of the flexor digitorum longus muscle (FDL) and the tibialis anterior-extensor digitorum longus (TA-EDL) muscles. The inhibition of TA EDL units was mainly induced by GS contraction. The FDL Ia units were inhibited by passive stretch of GS. It is suggested that GS tendon organs were responsible for the inhibition of TA EDL units and that GS primary endings were responsible for the inhibition of FDL units. Thus, there appears to be a high degree of specificity also in the inhibitory actions on the DSCT neurones.

There are 3 types of stretch receptors in mammalian skeletal muscle. The signals from each of these are relayed to the cerebellum through three largely separate groups of neurones in the Clarke column which is the origin of the dorsal spinocerebellar tract (DSCT). The signals of the second order DSCT cells are remarkably similar to the signals of the corresponding receptors (Jansen and Rudjord 1965, Jansen, Nicolaysen and Rudjord 1966, 1967). The main difference between the signals of the first and the second order neurones of the system is the irregularity of firing of the latter. Simply considered this irregularity only represents the introduction of "noise" into the signal, and teleologically one expects a better justification for the presence of the Clarke column synapse. One agreeable suggestion is that the signal can be modified by inhibitory interaction during the synaptic transfer. Inhibitory effects have been demonstrated for the DSCT cell (Laporte, Lundberg and Oscarsson 1956, Holmquist, Lundberg and Oscarsson 1956, Eccles, Oscarsson and Willis 1961, Hongo and Okada 1966). It has commonly been stated that inhibitory effects are particularly powerful between antagonistic muscles (Oscarsson 1965

reduction in firing frequency even with this appreciable load on TA EDL. A few units that were only weakly excited by stretch of GS were more powerfully inhibited from TA EDL. However on account of its transient nature and inconstancy the TA EDL effects on GS Ia units were not investigated in greater detail.

GS effects on FDL units Stretch of the triceps muscle regularly caused a reduction in the firing frequency of FDL units. An example of the effect is shown in Fig 2 A. The effect was strongest during and immediately after the dynamic stretch. During the following second there was some recovery of cell firing but the inhibitory effect remained as long as the GS stretch and this was the usual finding Similar observations were made for 7 of the 8 units of this kind that have been examined. Fig 2 C serves to illustrate that the inhibition was rather more marked during the dynamic phase of the stretch than during the ensuing static extension.

Fig 2 illustrates the firing pattern of a FDL unit during a GS twitch. There was a period of increased firing during the rising phase of the twitch which was followed by a period of inhibition during the later part of the twitch. Comparable observations were made in the two other units in which it was investigated. The timing in relation to the twitch of this inhibition in FDL units should be compared to that in TA EDL units illustrated in Fig 1 B.

An important factor must be considered before accepting the GS effects on FDL units as caused by true inhibition. The GS muscle lies over and intimately related to the FDL muscle. Taking the high sensitivity of particularly the primary endings of the muscle spindles into account one anticipates a certain degree of mechanical coupling between the two muscles That is to say that GS stretch may influence the FDL primary endings directly even though we tried to avoid this effect by the experimental arrangement. This has been investigated in one experiment in which the response of 9 FDL primary endings was determined at different lengths of the muscle with and without simultaneous stretch of the GS muscle up to approximately full physiological extension of the muscle Of the 9 FDL units the firing frequency of four of them was not perceptibly or only slightly affected by GS stretch. Four other units moderately increased their response when the GS muscle was extended, and only one unit showed some reduction in firing frequency on GS stretch and this effect was only seen at two of the five lengths at which it was examined. Thus, the conclusion was reached that there was some "mechanical coupling" between the GS and the FDL muscles in the present type of experiments, but this was usually in the direction of additional excitation of FDL primary endings on GS extension, and can therefore not explain the inhibitory interaction described above The other types of stretch receptors of skeletal muscle are less interesting in the present context. The existence of peripheral interaction between the GS and FDL muscle makes, however a quantitative study of the inhibitory effects less attractive

To avoid the mechanical interaction between the two muscles the effect on FDL I units of electrical stimulation of the central end of the GS nerves has also been examined. An example of the results is illustrated in Fig 3 The GS nerve was stimulated repetitively at a t of 110 sec with shocks slightly less than needed

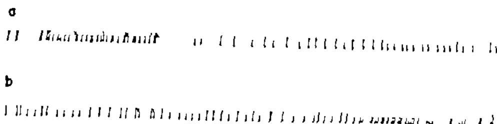


Fig. 5. Effect of repetitive stimulation of GS nerve on FDL unit. FDL muscle fully extended. Period of GS stimulation at 110/sec and intensity of about 90% of group I maximal indicated by continuous line above action potential record. GS nerve cut peripherally. Record a and b are continuous. Time marks 0.1 sec.

elicite a maximal group I volley. Very soon after the onset of stimulation the unit stopped firing for more than 200 msec and recovered gradually during the following second. The inhibitory effect, however, persisted during the 4.5 sec that the stimulation was continued and there was some rebound increase in firing after the end of the inhibition. Similar observations were made on all the seven units that have been examined so far. The inhibitory effect was only slight with shocks of less than 50 per cent of group I maximal strength and increased with increasing size of the shock. For two units a moderate further increase in the inhibition was found with shocks about 5 times group I threshold. At low rates of GS nerve stimulation (about 10/sec) the inhibitory effect was barely recognizable. It increased with increasing stimulus frequency up to 100/sec, but the steady state inhibitory effect was only slightly increased on further increase to 160/sec. Similar observations were made on the inhibition of TA EDL Ia units by electrical stimulation of the GS nerve.

These experiments with electrical stimulation of the GS nerve support the conclusion that mechanical coupling from the GS muscle does not account for the inhibition of the FDL units. The similarity in time course of the adequately and the electrically elicited inhibition suggests that also the former was mainly a group I effect and finally the prolonged period of steady inhibition appears to be suitable for a more detailed quantitative study of the interaction between excitation and inhibition in the DSCT. This will be dealt with in a subsequent communication.

Discussion

Two aspects which deserve some additional comments are raised by the observations presented. The first concerns the type or types of receptors responsible for the stretch evoked inhibition. Initially it should be stated that the present type of experiments does not permit conclusive statement about the origin of the inhibition. One may postulate a number of more complicated mechanisms which would explain the observations. So the aim of the discussion must be to find the simplest explanation that accounts for the experimental results so far obtained.

Three points about the GS inhibition of TA EDL Ia units appear to be particularly relevant. It occurred early during the twitch and lasted only into its falling phase

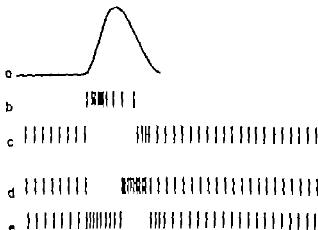


Fig. 4. Diagrammatic, tentative explanation of the time course of inhibition of TA-EDL units (c) and FDL units ( ) during a GS twitch ( ) b. Discharge pattern of GS tendon organs during the twitch. d. Discharge of GS primary endings during the twitch.

It was only weak or even absent during passive stretch of the GS muscle, and finally it was imitated by electrical stimulation of the GS nerve of group I strength. All three findings suggest that this inhibition originated in GS Golgi tendon organs. The moderate effect of passive stretch of the muscle is explained by the observation by Jansen and Rudjord (1964) that many of these tendon organs are only weakly or not at all excited by passi e extension. Tetanic contraction of the muscle on the other hand, is always a powerful tendon organ timulus and had regularly strong inhibitory effects n the present experiments (Fig 1 C)

The GS effects on the FDL Ia units presented a different pattern. The inhibition now occurred during the falling phase of the twitch, at a time when the primary endings of a muscle usually fire a igorous burst of impulses on being reextended. This period of inhibition was preceded by a period of excitation during the rising phase of a twitch. In this period th primary endings are silent as is well known. Furthermore, t was more pronounced during the dynamic phase of passive stretch (Fig 2 C) but commonly present also during maintained extension, and finally comparable inhibitory ffect was el cited by group I stimulation of the GS nerve. Considered together these bserv ions suggest that the GS primary endings are largely responsible for he nhibitory effects from this muscle on the FDL Ia units, but additional effects from the sec ndary endings are not excluded. A diagramatic illustration of the t tat e explanation of the origin and time course of the inhibitory effects is gi en Fig 4 It show a GS twitch (a) with the firing pattern of a GS tendon organ b and a primary ending (d) and the corresponding inhibition of a TA EDL unit ; nd FDL unit

Considering next the functional significance of the inhibition in the DSCT some relatively simple fact ppear t be established. Significant amounts of inhibition ca be produced by adeq uat stim lation of stretch receptors of other muscles than hat relaying through gi en DSCT ell. The degree of activation of the receptors needed t produce inl bitory effects are not greater than what may be exper o

occur during physiological activities such as walking. There appears to be a high degree of specificity also in the inhibitory actions on the DSCT cells. GS tendon organs inhibit ankle flexor units while its primary endings inhibit FDL units. Similar specificity has already been demonstrated for the excitatory actions on DSCT cells (Lundberg and Oscarsson 1956, Jansen and Rudjord 1965). This means that the signals mediated by the DSCT in units are not simply functions of length and rate of change of length of the appropriate muscle, but are instead rather more complicated functions of length, velocity and tension in a number of interrelated muscles. Most appropriately the DSCT signals should probably be described in relation to limb position, movement and degree of contraction in the various muscles. A very extensive piece of work remains before the full complexity of the situation has been revealed.

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Acta physiol. scand. 1967 70 369—388

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The Effect of DOPA on the Spinal Cord

5. Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors

By

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Abstract

Jankowska, E. M. G. M. Jukes, S. Lund and A. Lundberg. *The effect of DOPA on the spinal cord. 5. Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors* Acta physiol scand. 1967 *70* 369—388

In unanaesthetized spinal cats, injected with L-DOPA, volleys in the flexor reflex afferents (FRA) evoke a long-latency longlasting discharge in ipsilateral flexor and contralateral extensor motoneurones. It is postulated that this discharge is transmitted by a neuronal pathway which is inhibited in the normal acute spinal cat, presumably from the pathway which in this state transmits the short-latency effect from the FRA to motoneurones. The organization of the pathway released by an injection of DOPA has been analyzed by recording the discharges in efferents to flexors and extensors and with intracellular records from motoneurones. Combined stimulation of ipsilateral and contralateral FRA reveals a reciprocal organization in that either flexor or extensor motoneurones are activated. Transmission from the ipsilateral FRA to flexor motoneurones can be inhibited by volleys in the contralateral FRA, and transmission from the contralateral FRA to extensor motoneurones by volleys in the ipsilateral FRA. These inhibitory effects are neither produced postsynaptically in the motoneurones nor presynaptically by depolarization of primary afferents and are hence exerted at an interneuronal level. The organization of reciprocal innervation at an interneuronal level is discussed in relation to the primary afferent depolarization evoked in Ia afferent terminals after DOPA and to rhythmic alternating movements.

Intravenous injection of DOPA profoundly changes the effects within the spinal cord of stimulation of the FRA (flexor reflex afferents) The short latency effects normally evoked, e.g. depolarization in primary afferents and the synaptic action in motoneurones, are depressed and instead longlasting actions occur after a considerably latency (Andén *et al.* 1966a) Volleys in the FRA normally evoke a PAD (primary afferent depolarization) in their own but not in those of Ia afferents, however after DOPA a large PAD is evoked in Ia afferent terminals [illegible] 1966c) This was interpreted as essentially a release phenomenon [illegible]

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lated that DOPA (through liberation of transmitter from a descending noradrenergic pathway) inhibits transmission from the FRA *to the terminals of the FRA* and also releases from an inhibitory control transmission in a pathway from the FRA to the *terminals of Ia afferents*. This inhibitory control is normally exerted by activity in the first pathway.

The present paper is concerned with the organization of the pathway transmitting effects to motoneurones after DOPA. It will be shown that reciprocal innervation is achieved at an interneuronal level and that this permits a discharge of flexor or extensor motoneurones much as may be inferred from Graham Brown's ideas about 'half-centres'. Data concerning interneurones transmitting the late effects after DOPA will be reported in a following paper. Preliminary reports have been given (Jankowska *et al.* 1965; Lundberg 1965).

Methods

All experiments were made on unanaesthetized acute spinal cats, which were anaemically decorticated or decerebrated as described by Andén *et al.* (1966*a*). Intracellular recording from motoneurones was made with microelectrodes filled with a solution of 2 M potassium citrate. For other experimental procedures and drugs employed see Andén *et al.* (1966 *a*, *b*).

The following abbreviations are used: DOPA, L-3,4-dihydroxyphenylalanine; FRA, flexor reflex afferents; PSP, postsynaptic potential; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; DRP, dorsal root potential; PAD, primary afferent depolarization; Q, quadriceps; vast., vastus; sart., sartorius; Saph., saphenous; H, hamstring; PBSt, posterior biceps-semitendinosus; PB, posterior biceps; ABSm, anterior biceps-semimembranosus; AB, anterior biceps; ten., tenuissimus; Sur, sural; G-S, gastrocnemius-soleus; G, gastrocnemius; [illegible], soleus; Pl, plantaris; J, joint; FDHL, flexor digitorum et hallucis longus; tib. ant., tibialis anterior; SP, superficial peroneal; DP, deep peroneal; EDL, extensor digitorum longus; VR, ventral root; i, ipsilateral; co, contralateral; lat., lateral; cond., conditioning.

Results

1. *Synaptic actions evoked from the FRA after DOPA*

(*a*) *Efferent discharges*. Andén *et al.* (1966 *a*) briefly reported that after DOPA volleys in the FRA evoke a longlasting discharge in ipsilateral flexor motoneurones and in contralateral extensor motoneurones. Fig. 1 illustrates this with stimulation of muscle afferents of both hindlimbs and simultaneous records from flexor and extensor efferents on one side. Clearly the discharges are evoked from high threshold afferents, in the experiment of Fig. 1 exclusively from group III afferents. No late effects were ever caused by volleys in group I afferents, but in some experiments they appeared at stimulus strengths between 3 and 5 times threshold indicating effects from impulses in group II afferents. Fig. 1 illustrates the selectivity in activation of flexor and extensor motoneurones. When the ipsilateral nerve is stimulated (B—D) there is a massive discharge in efferents to the flexors but not in those to the extensors, while the reverse occurs on stimulation of the contralateral nerve (F—H). Stimulation of the ipsilateral FRA evoked discharges in efferents to the following muscles: sartorius (medial and lateral), gracilis, semitendinosus, posterior biceps, tenuissimus and tibialis anterior. Corresponding effects were evoked from the

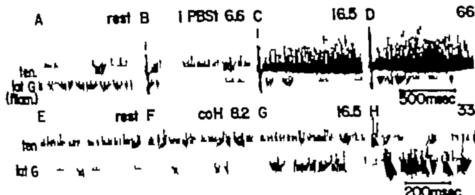


Fig. 1. Discharges in efferents to flexor and extensor muscles evoked by short train of volleys in ipsi- and contralateral afferents respectively. Upper traces—discharges in the nerve to tenuissimus. Lower traces—discharges in filament (A—D) or the whole nerve (E—H) to lateral gastrocnemius. The resting discharges in the tenuissimus nerve is in γ-efferents. The afferents from IPBSt and coH were stimulated with increasing strengths of current, indicated in multiples of thresholds for group I fibres. The records were taken after intravenous injection of 100 m/kg of DOPA.

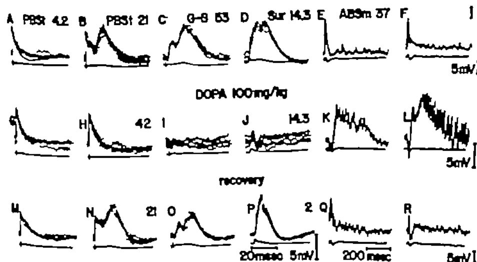


Fig. 2. Effects of DOPA on the EPSPs evoked in flexor motoneurones by volleys in FRA. The upper traces are intracellular and the lower from the L7 dorsal root entry zone. A—F, G—L and M—R are from three different PBSt motoneurones and were taken before, few minutes after and about two hours after an injection of 100 mg/kg of DOPA. The stimulus strengths, expressed in multiples of thresholds were the same for all three motoneurones unless otherwise indicated and are shown above each column of records. The four left columns show the effect of single volleys at the fast sweep speed (time calibration below P). short train of volleys were employed to obtain the records in the two right columns (time calibration below Q). Voltage calibration refers to intracellular records A—F, G—L, M—P and Q—R respectively.

contralateral FRA in efferents to semimembranosus, anterior biceps, rectus, vasto-crureus, gastrocnemius-soleus and plantaris. Hence muscles which are identified by other criteria as flexor or extensors (Sherrington 1910, Eccles and Lundberg 1959) are activated from the ipsilateral and contralateral limb respectively. No

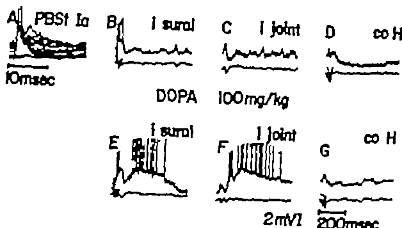


Fig 3. Modification by DOPA of the effect evoked from the FRA in flexor motoneurone. The intracellular records (upper traces) are from the same PBSt motoneurone with the homonymous Ia EPSP shown in A and PSPs evoked by a train of volleys in the FRA in B—G. Lower traces are from the L7 dorsal root entry zone. Time calibration below A refers to A and below G to B—G. Voltage calibration refers to intracellular records.

discharges were evoked in the nerves to the toe muscle FDHL and EDL in the two experiments in which recording was made from these nerves. Further experiments are required to find out if these muscles participate in the reflex.

b) *Intracellular recording*. This has been employed to find out the mechanism by which the late discharges are evoked. Fig 2 illustrates the sampling procedure. The records are from three flexor motoneurones, A—F before DOPA, G—L soon after the injection of DOPA and M—R after the recovery from the effect of DOPA. After DOPA there is the expected depression of short latency effects from high threshold muscle afferents and cutaneous afferents (H—J) (cf. Andén et al. 1966a) and a longlasting EPSP appears (K—L) which was not present before DOPA (E, F). Two hours later the effect of DOPA has subsided. The short-latency effects from the FRA have reappeared (N—P) and there are no late effects in the slow records (Q, R). Since in experiments of the type shown in Fig 2 virtually all flexor motoneurones received the late EPSP it is inferred that the short-latency effects before and the long latency effects after DOPA are evoked in the same motoneurones. This conclusion has been confirmed with recording from the same motoneurone before and after DOPA as illustrated in Fig 3. Such recordings are difficult to obtain because the injection of DOPA gives rise to considerably increased vascular pulsations, usually leading to damage or loss of the motoneurones impaled by the microelectrode. The late EPSPs were evoked in flexor motoneurones from the ipsilateral FRA and in extensor motoneurones from the contralateral FRA (Fig 8) and in motoneurones with fast and slow afterhyperpolarizations indicating that the effects after DOPA reach motoneurones of both fast and slow muscles (cf. Eccles et al. 1958). The duration of the EPSP evoked from the FRA after DOPA can vary within wide limits. Usually it lasts between 0.4 to 1 sec but evidence suggesting much more prolonged actions has frequently been obtained both with recording

Fig. 4. After-discharge in efferents to flexor muscle. Upper traces are records from branch of the PI nerve the lower traces are from the L7 dorsal root entry zone. Stimulation of the PI nerve at the strengths indicated, with single shocks in A and D and with short trains in B, C, E and F. A—C are control records taken before DOPA, D—F were obtained 10 min after the injection of DOPA. Observe the decreased effect of reflex volleys and the shortening of the after-discharge.

A PI 50 B 25 C 50
DOPA 100mg/kg
D PI 50 E 25 F 50
20msec 500msec

from peripheral nerves and from motoneu nes ... discharges lasting for more than 30 sec have occasionally been record d

c) *Deformation from after-discharge* Longla ... are found not only after DOPA but also ... DOPA in the acute unanaesthetized spinal cat (cf. Creed *et al.* 1932 ...) ... in flexor efferents (B, C) and Fig. 5 longla ... extensor motoneurones. These late effects ... from the late effects after DOPA ... are always preceded by pronounced ... the short-latency effects are depressed and the ... without any preceding short-latency effect ... tial of synaptic actions in Fig. 5 represents an ... FRA pathway which slowly subsides. The time ... the discharge after DOPA differ in a character... gradual onset attaining a maximum in 200–400 ...

A ABSm 4 B 40
PBSt
motor
5mV
D CP 42 E 42 F
ABSm
motor
... msec 5mV

Fig. 5. Lengthening PSP ... Upper traces ... from the L7 dorsal root ... EPSPs and IPSPs evoked ... with the ... [illegible]

charge gradually subsides from an initial maximum. Indirect evidence suggests that the longlasting effect after DOPA does not represent an increase in the after-discharge but is an entirely separate phenomenon (cf. Jankowska et al. 1967). In fact on those few occasions when injection of DOPA did not lead to the appearance of any late effects in primary afferents and motoneurones, it was observed that there was a suppression of the after-discharge (E, F Fig. 4) as would rather be expected from the postulated inhibitory effect on the short latency FRA pathway.

It is, however, occasionally difficult to classify the origin of effects in motoneurones after DOPA, particularly when volleys in the FRA evoke a motoneuronal discharge after short latency. Probably these are, at least partly, caused by activity remaining in the short latency pathway but it is possible that the pathway released by injection of DOPA may sometimes transmit actions with a latency that not much exceeds that of the short-latency pathway (Fig. 9 D).

2. *Reciprocal innervation*

a) *Efferent discharges.* In association with the late longlasting discharge there is inhibition of transmission to antagonist motoneurones. In Fig. 6 the discharge in tenuissimus efferents is evoked from ipsilateral high threshold muscle afferents (B) and in efferents to lateral gastrocnemius from contralateral high threshold muscle afferents (C). D—I show interaction of these volleys from the two hindlimbs at different intervals with the ipsilateral stimulation leading in D—F and the contralateral in G—I. With strong stimulation of the ipsilateral nerve (66 times threshold) there is inhibition of the discharge to the extensor while the discharge to the flexor remains. An extensor discharge occurs only in F at a very long conditioning testing interval and then only after the cessation of the flexor discharge. In J—L the ipsilateral nerve is stimulated at a weaker strength and now the discharge in extensors evoked from the contralateral FRA dominates although somewhat reduced the discharge in the lateral gastrocnemius efferents remains (K) while the discharge in flexor efferents is almost completely inhibited. At the longer interval in L the flexor discharge occurs with a delayed onset after the cessation of the main discharge in the extensor efferents. In conclusion the discharge in flexor efferents can be accompanied by inhibition of transmission to extensor motoneurones and vice versa, under other conditions the discharge in extensor motoneurones may be associated with inhibition of transmission to flexors.

b) *Intracellular recording.* An interesting feature in Fig. 1 and in Fig. 6 is that the discharges whether evoked in flexor or extensor motoneurones are not necessarily accompanied by a reduction of the resting discharge in antagonist motoneurones. This suggests that the reciprocal effects, disclosed by the interaction experiment (Fig. 6) are not necessarily caused by postsynaptic inhibition of motoneurones. However as far as α-motoneurones are concerned no definite conclusion can be drawn because the discharges in Fig. 1 and 6 are mainly in γ-efferents. Nevertheless this finding necessitated a further analysis with intracellular recording. The records in Fig. 7 are from a PBSt motoneurone and a test EPSP is evoked from ipsilateral

Fig. 6. Interaction of effects evoked from the ipsi- and contralateral FRA. Simultaneous recording from nerves to [illegible] (upper traces) and lateral gastrocnemius (lower traces). A, resting discharges. B and C, responses to train of volleys in the ipsilateral PBSt and contralateral H nerves respectively. The duration of the tetanic stimulation is marked in each case by line below the lower traces, shorter for l PBSt and longer for coH. D—L show effects of combined stimulation [illegible] different intervals of the ipsi- and contralateral nerves. Note different strengths of stimulation of the PBSt nerve (66 and 33 times threshold in D—F and J—L respectively) and reversed sequence of stimulation of the l PBSt and coH in D—F and G—J. DOPA (100 mg/kg) had been given.

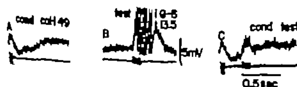


Fig. 7. Inhibition from the contralateral FRA of transmission from the ipsilateral FRA to flexor motoneurones. Upper traces are intracellular from PBSt motoneurone and the lower traces are from the L7 dorsal root entry zone. DOPA (100 mg/kg) had been given. B shows the [illegible] EPSP evoked by volleys in ipsilateral FRA, A the effect of volleys in the contralateral FRA and C the effect of the combined stimulation of the ipsi- and contralateral nerves. Voltage calibration refers to the intracellular records.

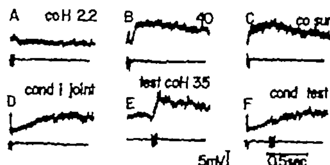


Fig 8. Inhibition from the ipsilateral FRA of transmission from the contralateral FRA to an extensor motoneurone Upper traces are intracellular from a G-S motoneurone and lower traces are intracellular from G-S motoneurone and lower traces are from the L7 dorsal root entry zone. DOPA (100 mg/kg) had been given. The effect of stimulation of contralateral nerves is shown in A—C and E and of the ipsilateral joint nerve in D F was obtained with combined stimulation of ipsi- and contralateral nerves. Voltage calibration refers to the intracellular records.

high threshold muscle afferents in B. A conditioning volley from the contralateral side gives an early EPSP followed by a fairly longlasting IPSP Combined stimulation in C results in a profound depression of the test EPSP only the initial part of the EPSP remains. This finding may be somewhat difficult to interpret because of the fact that the conditioning volleys in this neurone give an IPSP The fact that the initial part of the EPSP remains, whereas the later part of the longlasting EPSP is wiped out, strongly suggests that the depression is caused not by post synaptic changes in the motoneuronal membrane but by inhibition of transmission of the synaptic excitatory action to the motoneurone. This inhibition could be either at a primary afferent level or at an interneuronal level The former alternative can be excluded because the inhibition occurs at conditioning testing intervals exceeding the duration of the crossed DRP evoked by the conditioning volleys. It must be recalled that only the first component of the crossed DRP which in the present experiments lasted 100—150 msec is due to a PAD in the FRA (cf R. M. Eccles et al. 1964a, b) The late crossed DRP is entirely caused by a PAD in Ia afferent terminals (Jankowska et al. 1966) and would not give any inhibition of transmission from the ipsilateral FRA to flexor motoneurones at primary afferent level.

Corresponding findings have been made with respect to transmission from the contralateral FRA to extensor motoneurones as illustrated in Fig 8. Volleys in contralateral cutaneous afferents (C) and high threshold muscle afferents (B) give a very longlasting EPSP Volleys in the ipsilateral high threshold joint afferents evoke an IPSP after short latency which subsides much faster With combined stimulation of ipsilateral and contralateral nerves (test alone shown in E) there is complete inhibition of transmission from the contralateral side (F) Clearly with this long conditioning-testing interval, the inhibition cannot be caused by a PAD (cf above) furthermore it occurs after an interval when the IPSP has nearly subsided and very little conductance change would be expected. For a final proof that the inhibition, illustrated in Fig 7 and 8 is exerted at an interneuronal level it would be desirable to perform conductance measurements in the motoneurones.

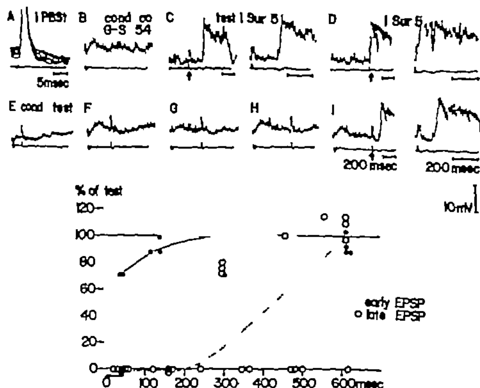


Fig. 9. The effect of conditioning volleys in the contralateral FRA on early and late components of the EPSP evoked from ipsilateral FRA in flexor motoneurone. Upper traces are intracellular from PBSt motoneurone and lower traces are from the L7 dorsal root entry zone. DOPA (100 mg/kg) had been given. The effect of conditioning volleys in the contralateral FRA is shown in B. The test responses from the ipsilateral FRA, shown in C and D, were evoked by single volleys in the sural nerve. E—I illustrate the effect of combined stimulation at different intervals of the contra- and ipsilateral nerves. The double records in C, D and I were obtained simultaneously at two different sweep speeds, the arrows marking the beginning of the expanded right sweep, and the time calibration corresponding to 200 msec in all the records in B—I. Voltage calibration is for intracellular recording. The graph shows the amplitudes of the early and late components (see records in C, D, I) of the EPSPs from FRA, conditioned by volleys in co FRA, plotted as function of the interval between conditioning and testing stimulation and expressed as percentages of control test values. Line below the ordinate indicates duration of the conditioning repetitive stimulation.

However fairly conclusive evidence that a concealed conductance change cannot be the explanation is given by the data illustrated in Fig. 9. In this case hardly any synaptic effects are evoked from contralateral afferents (B). The testing volley in the sural nerve gives an EPSP with a double configuration. There is an initial EPSP after a short latency and after a varying interval, the characteristic longlasting EPSP appears. In different tests the interval between early and late EPSPs varied between the extremes shown in C where it exceeds 250 msec in D where the longlasting EPSP arises almost from the summit of the initial EPSP. The series of records in E—I and the curve below show the effect of combined stimulation: complete inhibition of the late EPSP with little or no effect on the early

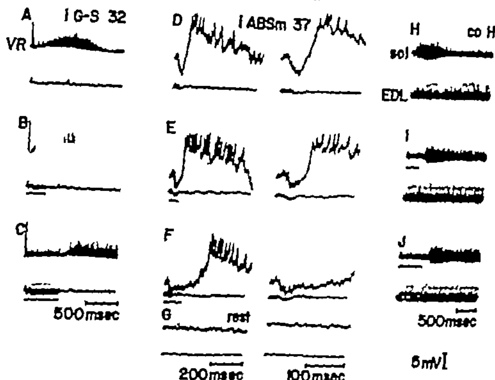


Fig. 10. Delay in the onset of the effect evoked from the FRA with prolongation of the repetitive stimulation. A—C, records from a filament of the L7 ventral root (upper traces) and H—J from nerves to soleus (upper traces) and extensor digitorum longus (lower traces) muscles. The upper traces in D—G are intracellular from a PBSt motoneurone, the lower traces in A—G are from L7 dorsal root entry zone. The double records in D—G were obtained simultaneously at different speeds. DOPA (100 mg/kg) had been given. The FRA effects were evoked from ipsilateral gastrocnemius-soleus, ipsilateral anterior biceps-semimembranosus and contralateral hamstring nerves, as indicated for A—C, D—F and H—J respectively. The lines of different lengths below the records show the duration of the repetitive stimulation, whenever the shock artefacts are difficult to distinguish. Voltage calibration refers to the intracellular records. Time calibrations are for the respective vertical columns.

LPSP. It is therefore postulated that the inhibition of the late EPSP is not caused by a conductance change in the motoneurone but occurs at an interneuronal level; the curve in Fig. 9 shows the duration of the inhibition. As with the longlasting synaptic excitation there is complete recovery only after about 600 msec. An interesting feature in the curve is the almost all or none appearance of the late EPSP in the intervals between 290—620 msec. In 14 trials the EPSP failed to appear six times but on the eight occasions when it appeared it had almost the same size each time.

The effective reciprocal inhibition is presumably caused largely by this interneuronal inhibition, but in individual motoneurones postsynaptic inhibition of motoneurones (cf. Fig. 7 and 8) may contribute. The origin of this postsynaptic inhibition in antagonist motoneurones is not known. It may be due to incomplete inhibition by DOPA of transmission in the short-latency pathway or Renshaw inhibition secondary to the motoneuronal discharge. The third possibility is that,

associated with the pathway giving the longlasting motoneuronal excitation, there is a pathway giving reciprocal postsynaptic inhibition in antagonist motoneurones. We do not favour the latter hypothesis for the reason that in many motoneurones there was no evidence of IPSPs evoked from the FRA of the other limb to that giving the late EPSP (Fig. 5 G and Fig. 9 B). It would be surprising if a reciprocal inhibitory pathway existed but would fail to give any effect although the excitatory action is so pronounced.

Many observations have been made with similar conditioning-testing experiments in acute spinal cats without DOPA. No evidence was found suggesting inhibition of the short latency effects from the FRA at intervals exceeding the duration of the evoked primary afferent depolarisation. This shows that the inhibitory process discussed above is not active on the short-latency pathway (cf. the Fig. 9) and that its function seems to be exclusively associated with transmission in the pathway giving the longlasting discharge after DOPA.

3. *Other inhibitory phenomena*

Andén *et al.* (1966 c) demonstrated that the late PAD evoked from the FRA in the terminals of Ia afferents after DOPA could also be inhibited by volleys in the FRA. It was postulated that the inhibition is caused by activity in the short-latency pathway from the FRA to the terminals of the FRA and that this process explains why transmission in the pathway to the terminals of Ia afferents is inhibited in the acute spinal cat without DOPA, when activity in the short latency FRA pathway is easily evoked. The similarity between the longlasting effects to primary afferents and to motoneurones suggested that a similar situation could exist for transmission to motoneurones. The records from muscle efferents in Fig. 10 (A—C and H—J) illustrate that prolongation of the repetitive stimulation delays the onset of the late discharge, which occurs only at the end of the stimulation. The intracellular records D—F show that the prolongation of the latency is caused by a delayed onset of the EPSP in the motoneurones. Thus transmission of the longlasting effects after DOPA to motoneurones can be both evoked and inhibited from the same afferents. Indirectly this strongly suggests a similar organization to that for primary afferent terminals, i.e. that the long latency pathway from the FRA to motoneurones which is active after DOPA is normally inhibited by activity in the short latency pathway from the FRA.

When DOPA is given after a previous injection of a *monoamine oxidase inhibitor* (Nialamide) large spontaneous fluctuations of membrane potential are commonly seen in the intracellular records from motoneurones. The effects of volleys in the FRA depend upon the level of membrane potential at the time illustrated in Fig. 11. At high potential (A, B) there is no effect, at low potential (E, F) a large hyperpolarization is produced and at intermediate levels (C, D) a moderate hyperpolarization occurs. In each case the membrane potential becomes polarized to the same level. The hyperpolarization could be an IPSP which does not appear in A because the membrane is already at the equilibrium potential for the IPSP but this seems unlikely for similar effects can be seen in motoneurones with membrane potentials far from the equilibrium potential. We suggest that the hyperpolarization is due

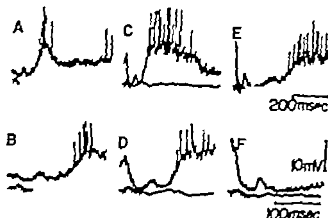


Fig. 11 Intracellular records (upper traces) from PBSt motoneurone after administration of 10 mg/kg of Nialamide and 100 mg/kg of DOPA. Lower traces are from the L7 dorsal root entry zone. All the FRA responses were evoked from the ipsilateral joint nerve. The corresponding records in the upper and lower row (A—B, C—D, E—F) were taken simultaneously at different sweep speeds. Voltage calibration refers to intracellular recording

to the removal of excitatory synaptic bombardment arising at an interneuronal level. Investigations by Jankowska *et al.* (1967) indicate that the interneurones responsible are those transmitting the longlasting discharge from the FRA. Hence the hyperpolarization suggests the existence of an inhibitory action of the FRA on these interneurones.

4 Other types of excitatory responses

The ipsilateral flexor and the contralateral extensor reflex described in the first section dominate the reflex picture after injection of DOPA, but other types of response are occasionally seen, e.g. as illustrated in Fig. 12 in which simultaneous records are shown from the nerves to tibialis anterior and soleus. Before DOPA (A—D) there is a marked after-discharge in the flexor efferents evoked from the ipsilateral nerves but no discharge in either flexor or extensor nerves is evoked from the contralateral limb. After DOPA (E—H) volleys in the contralateral nerve give the expected longlasting discharge in extensor efferents (H) and volleys in the ipsilateral nerve a corresponding effect in the flexor efferents (F—G). However the unusual feature is that also from the ipsilateral nerves a discharge is evoked in the extensor efferents. This effect is not altogether exceptional and has been observed

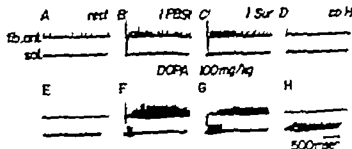


Fig. 12. Ipsilateral extensor reflex after DOPA. As in Fig. 1 but upper traces are from the nerve to tibialis anterior (tib. ant.) and lower traces from the nerve to soleus (sol.). Stimulation was supramaximal for group III afferents



Fig. 13. Facilitation of the late EPSP from ipsilateral FRA by conditioning volleys in contralateral FRA. Test response evoked from the joint nerve shown alone in B. I tracellular records (upper traces) from PBSt motoneurone. Lines below lower traces (from L7 dorsal root entry zone) show the duration of the test stimulation of the joint nerve. DOPA (100 mg/kg) had been given. Voltage calibration refers to the intracellular recording.

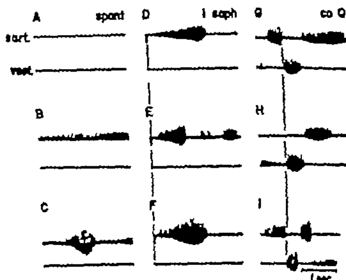


Fig. 14. Reciprocal discharges in nerves a flexor (sartorius) and extensor (vastus) muscles, evoked from co FRA G H and I. All records were obtained after administration of 10 mg/kg of Nialamide and 100 mg/kg of DOPA.

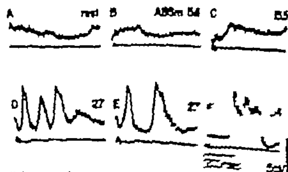


Fig. 15. Intracellular records (upper traces) ... 10 mg/kg of Nialamide and 100 mg/kg of ... after administration ... motoneurone was stimulated ... anterior biceps ... lular recordings. ... calibration refers to the ...

also in nerves to fast as well as to slow muscles. A corresponding phenomenon is illustrated in Fig 13 with intracellular recording from a PBSt motoneurone. B shows the longlasting synaptic effect from ipsilateral high threshold joint afferents but there is no effect in A from contralateral cutaneous afferents. However the combined stimulation in C reveals that transmission from ipsilateral nerves is facilitated from the contralateral side. Hence in this case the dominating effect from the contralateral side is excitatory instead of the more common inhibitory described in section 2

As described above when DOPA is given after Nialamide the resting potential may display spontaneous fluctuations. Under these conditions there may occur resting discharges in efferents and interesting responses are also evoked when the FRA are stimulated Fig 14 is from such an experiment and shows that sequences of alternating discharges may appear in flexor and extensor efferents. The sample records in this figure are shown to illustrate a great variability The reflex effects evoked from the contralateral limb clearly can be described as spinal stepping Also in the experiment of Fig 15 DOPA was given after pretreatment with Nialamide. There are in this case only slight resting fluctuations (A) but with stimulation of high threshold muscle afferents at increasing strength (B—F) a rhythmic process is revealed. A small late wave appears in C but in D and E at 27 times threshold the EPSP is intermittent, appearing in three or two waves. With further increase of the stimulation strength this phenomenon disappeared and there is in F only one late EPSP wave Presumably the appearance of the first phase is now prevented by the operation of the inhibitory mechanism described in section 3

Discussion

In many respects the late longlasting discharge evoked from the FRA in motoneurones after DOPA parallels the effect evoked from the FRA on the terminals of Ia afferents after DOPA (Andén et al. 1966 c) and the mechanism may be analogous, i.e. transmission in one spinal pathway may be released from an inhibitory control

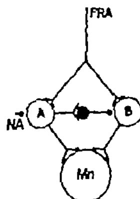


Fig 16. Schematic representation of neuronal pathways transmitting excitatory action to motoneurones (M) from the FRA. Excitatory terminals are indicated by two branches, inhibitory by filled circles. Termination of inhibitory interneurones on cell body merely indicates inhibitory action and there is no commitment as to whether the inhibition is postsynaptic on cell bodies or presynaptic on terminals of interneurones. Pathway A is activated in the acute spinal cat (without DOPA) and inhibits pathway B so that no effect is transmitted in this route. After DOPA transmission in pathway A is partially or completely inhibited (by liberation of transmitter from a descending noradrenergic pathway) thereby removing the inhibition of pathway B through which the late longlasting EPSP is evoked in the motoneurone. A single interneurone in the diagram may represent chain of inter neurones.

exerted from another spinal pathway This hypothesis is outlined in the diagram of Fig 16 with A representing the short-latency pathway from the FRA to motoneurones and B the pathway transmitting effects after DOPA. The inhibitory connection from A to B explains why pathway B is normally closed in the acute spinal cat and why after DOPA, which inhibits pathway A through liberation of transmitter from a descending noradrenergic pathway (cf. Andén *et al.* 1966a, b) pathway B is released as activity in A decreases. This hypothesis also explains why inhibition of the late discharge can be evoked by volleys in the afferents from which the discharges are evoked (Fig 10) Other evidence supporting the existence of an inhibitory interactive connection from pathway A to B has been given above (Fig 11 15) but these results do not require any further comments here, since very similar findings in relation to the pathways to primary afferent terminals have already been discussed in detail (Andén *et al.* 1966c, Jankowska *et al.* 1966)

The hypothesis schematically shown in Fig 16 will explain our findings but is more difficult to prove than that of alternative pathways from the FRA to primary afferent terminals (Andén *et al.* 1966c) The difficulty stems from the fact that both pathway A and B in Fig 16 converge onto the same neurones and exert the same synaptic action, whereas the effects from the FRA on primary afferent terminals before and after DOPA are exerted on terminals of different types of primary afferent. Merely on the basis of recordings from motoneurones it is difficult to exclude absolutely that the changes which occur after DOPA are not caused by a functional change in the pathway transmitting the short-latency effects before DOPA. However evidence has been presented suggesting that the discharge after DOPA should be differentiated from the after-discharge that may follow the short latency effects from the FRA in the acute spinal cat without DOPA (Fig 4 and 5) It is also difficult to imagine how DOPA could have changed the function in a pathway to enhance the after-discharge while suppressing the initial discharge. Recordings from interneurones strongly suggest that discharges after DOPA are mediated by interneurones, which normally do not transmit the after-discharge (Jankowska *et al.* 1967)

The d'emblée character of the flexor reflex in the acute spinal preparation has been contrasted with th recruiting type n the crossed extensor reflex (cf. Creed *et al.* 1932) However t was believed that the recruitment rather represented an artefact caused by a conflict between inhibition and excitation (p. 126, Creed *et al.* 1932) The reflexes evoked from the FRA after DOPA, on the other hand, are probably examples f genuine recruiting reflexes. The intracellular recordings from motoneurones show that the longlasting discharges are caused by sustained EPSPs. The amplitude of the EPSPs may be considerable, 10—20 mV and there is no obvious reason to assume that the synaptic terminals are differentially located from those giving the monosynaptic EPSPs or short-latency synaptic actions from the FRA. Presumably the long duration of the effect is almost entirely explicabl by the long duration of the ynaptic bombardment. The problem of how th interneuronal pathway can give such longlasting discharges will be discussed n report on interneuronal acti ity after injection of DOPA (Jankowska *et al.* 1967)

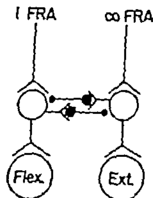


Fig. 17 Schematic representation of the principle organization of the neuronal pathway transmitting excitatory action to motoneurones after an injection of DOPA (pathway B in Fig. 16). Conventions as in Fig. 16.

The main part of the present paper has been devoted to the analysis of the functional organization of the pathways giving the longlasting discharges after DOPA (B in Fig. 16). The dominating reflex pattern is an ipsilateral flexor reflex and a crossed extensor reflex. The recording from efferents to many different muscles has revealed a parallelism with the short-latency effect in motoneurones in the acute spinal cat which was the basis for Sherrington's original classification of muscles as flexors and extensors (Sherrington 1910; Eccles and Lundberg 1959). We have briefly reported that other reflex actions may occur: both ipsilateral extension and crossed flexion. The conditions under which these effects appear have not been investigated in detail, the reason being that our main aim was to study the reciprocal relationship between flexor and extensor discharges, which is most clearly demonstrated with simultaneous recording from flexor and extensor efferents when discharges are evoked by interacting volleys in the FRA of the two hindlimbs. The analysis with intracellular recording from motoneurones has shown that transmission of the late longlasting EPSPs to flexor motoneurones can be very effectively inhibited by volleys in the contralateral FRA and, *vice versa*, that transmission of corresponding synaptic effects from the contralateral FRA to extensor motoneurones can be inhibited from the ipsilateral FRA. This inhibition may occur at conditioning testing intervals when EPSPs of other types are not influenced from the conditioning volley (Fig. 9). One can therefore exclude the possibility that the main cause of the inhibition is postsynaptic at the motoneuronal level. The inhibition must occur at an interneuronal level in the pathway transmitting the longlasting EPSP from the FRA to motoneurones because the duration of the inhibitory effect by far outlasts the PAD evoked in the contralateral FRA (cf. R. M. Eccles *et al.* 1964a, b). It is of course very relevant for our present problem that, although the crossed DRP evoked from the FRA has a very long duration, only the initial component is caused by PAD in the terminals of the FRA, while the late component is exclusively in Ia afferent terminals (Jankowska *et al.* 1966). Our conclusions, summarized in the diagram of Fig. 17, are based entirely on the present findings but is also strongly supported by the results obtained with recording of the discharges of interneurones (Jankowska *et al.* 1967).

In most reflex pathways reciprocal postsynaptic inhibition of antagonist motoneurones is an essential component of the reflex. No such inhibitory pathway is included in the diagram of Fig 17 although inhibitory postsynaptic actions may occur from the limb contralateral to that providing the excitatory action (Fig 7 and 8) When this inhibition occurs it may represent a remaining action through the short-latency pathway from the FRA or else Renshaw inhibition secondary to the activation of antagonist motoneurones. In many motoneurones, however there is no sign of postsynaptic inhibition from the opposite limb (Fig 3 and 9) and this strongly suggests that reciprocal postsynaptic inhibition of motoneurones is not a requisite part of the reflex.

Reciprocal postsynaptic inhibition of motoneurones has the eminent advantage that it acts on all excitatory effects converging onto motoneurones of antagonists. Reciprocal inhibition of interneurones according to the scheme in Fig. 17 prevents excitation of the motoneurone only from this particular pathway so that the excitatory action is evoked either in flexor or extensor motoneurones. This type of reciprocal inhibition obviously cannot influence other excitatory actions converging onto the motoneurones, and it is important to consider the limitation of a reciprocal inhibition operating on interneurones. Antagonist motoneurones may be influenced by other reflex actions but it should be noted that both the Ib pathway and the short latency FRA pathway are inhibited when the long-latency pathway operates (Andén *et al* 1966a.) The Ia reflex, on the other hand, could provide a complication. For example, during a flexor reflex through pathway B in Fig 16 the flexion of the limb would cause a discharge of Ia impulses from extensor muscles that, in the absence of reciprocal postsynaptic inhibition, could give excitation of extensor motoneurones. From this point of view it is of considerabl interest that corresponding with the late discharges to motoneurones there is a longlasting depolarization in the central terminals of Ia afferents (Andén *et al.* 1966c) Hence t is possible that presynaptic inhibition of transmission from Ia afferents to the motoneurones may prevent reflex activation of antagonists or in other words, serve as a substitut for postsynaptic reciprocal inhibition of motoneurones. Further investigations of this mechanism are required, a special challenge being that the organization of these connections from the FRA to Ia afferent terminals seems to have a functional meaning, while it is difficult to understand the significance of many of the pathways to primary afferent terminals.

The interneuronal organization in the diagram of Fig 17 seems eminently suited to subserve rhythmic alternating movements of which stepping and the scratch reflex are the examples best known (Sherrington 1906 Creed *et al* 1932) To illustrate the mechanism for production of alternate activation by the network in Fig 17 it is assumed that the two interneuronal pools do receive an pproximately equal excitatory action However a knife-edge balance with an equal discharge in both interneuronal pools cannot be maintained any dominance, however slight, of activity in one of the terneuronal pools increases the inhibition on the other pool and hence leads to a decreased inhibition of the already dominating pool. The change

will proceed until there is maximal activation of this pool and maximal inhibition of the other. With maintained activation there is accumulation of refractoriness, the reciprocal inhibition decreases and the other interneuronal pool takes over. *Hence an alternate activation will occur as long as the excitatory action is exerted* on the two interneuronal pools. Theoretical aspects on a neuronal network of this type have recently been discussed by Harmon (1964) and by Harmon and Lewis (1966).

Graham Brown (1911) demonstrated that spinal stepping may persist after *complete deafferentation of the limb exhibiting the movement.* He concluded that the alternating rhythmic movements of the spinal animal are essentially a central spinal process. In his hypothesis about 'spinal half-centres'—one giving excitation to flexor, the other to extensor muscles—Graham Brown postulated that each 'half-centre' could inhibit the other. In the original definition the 'primary half-centres' *denoted the motoneurones (Graham Brown, 1911, 1912)* and the possibility was discussed that reciprocal innervation might depend not only on opposite effects from primary afferents to motoneurones of antagonist muscles (Sherrington, 1906) but also on mutual inhibition between the two 'primary half-centres'. The term 'interposed half-centres' was used to describe the interneurones intercalated *between primary afferents and the 'primary half-centres'. Graham Brown postulated* mutual inhibition between those 'interposed half-centres' and also inhibition from each 'interposed half-centre' to the antagonist 'primary half-centre'. In a later publication the 'half-centres' included both the 'interposed' and 'primary half-centres' (Graham Brown, 1924).

The interneuronal network schematically shown in Fig. 17 could represent the neuronal correlate of the 'interposed half-centres', but there is no evidence for the other types of 'half-centre' inhibition postulated by Graham Brown. *It is not known* if the 'half-centre' organization is unique for the reflex pathway that is disclosed after the injection of DOPA or whether it occurs also in other reflex pathways with *double reciprocal organization. At present this cannot easily be tested because* of the difficulty of excluding that an inhibition evoked from contralateral nerves is presynaptic, caused by the crossed PAD (R. M. Eccles *et al.*, 1964 a, b). However, it is noteworthy that volleys in the contralateral FRA evoke IPSPs in some dorsal horn interneurones which receive excitatory action from the ipsilateral FRA (Hongo *et al.*, 1966).

Further experiments are required in order to decide what role the long-latency reflex actions which are revealed after DOPA may play in progression and other rhythmic alternating movements. The main relevance of the present work lies in the demonstration of the organization of the interneuronal connections shown in Fig. 17 through which *the spinal cord* is programmed at a segmental level for rhythmic alternating movements. The double reciprocal organization of these connections makes them particularly interesting in relation to stepping. Presumably stepping can be evoked through this network not only by reflex action from primary *afferents* but also by descending volleys and probably also by autochtonous activity in the

interneurones. It is therefore of interest that stepping can be evoked by stimulation of the cut surface of the cervical spinal cord (Roaf and Sherrington 1910 Sherrington 1910) and from an isolated region in the spinal cord by administration of a volatile anaesthetic—the 'narcosis progression' (Graham Brown 1913 1914)—in all likelihood by local depolarization of the interneurones. Stepping could ensue not only if both interneuronal pools, giving excitation to flexor and extensor motoneurones respectively were activated (cf. above) but also if only one of these pools was activated e.g. from supraspinal centres. Alternating activation could then follow reflex activation of the antagonist pool. In this connection the probable participation of secondary spindle afferents among the FRA is of interest, particularly in relation to the fact that static γ-motoneurones (cf. Matthews 1964 Appelberg *et al.* 1966) are reflexly activated after DOPA (Grillner *et al.* 1967)

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The Effect of DOPA on the Spinal Cord

6. Half-centre organization of interneurones transmitting effects from the flexor reflex afferents

By

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Abstract

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In unanaesthetized spinal cats systematic microelectrode exploration has been made in the lumbosacral spinal cord in order to find interneurones that may transmit the late longlasting reflex effects that volleys in the FRA (flexor reflex afferents) evoke after an intravenous injection of DOPA. Interneurones that may transmit these late effects are found in the lateral part of Rexed's layer VII and three main types are identified A) cells activated from the ipsilateral FRA and inhibited from the contralateral FRA, B) cells activated from the contralateral FRA and inhibited from the ipsilateral FRA, C) cells activated from both the ipsilateral and contralateral FRA. The receptive fields and other criteria employed suggest that type A and B transmit excitatory action to flexor and extensor motoneurones respectively and type C depolarization to I afferent terminals. The functional organization of these pathways is discussed mainly in relation to the mechanisms responsible for the long latency and duration of the discharge.

In previous papers of this series it was shown that after an intravenous injection of DOPA volleys in the flexor reflex afferents (FRA) have late longlasting reflex effects on the terminals of primary afferents and on motoneurones (Andén *et al.* 1966b Jankowska *et al.* 1966 1967). This paper is based on extracellular records made from those interneurones which appear to be concerned with the transmission of the reflex effects evoked from the FRA after the injection of DOPA. Special attention has been given to the reciprocal organization of effects from the two hindlimbs on many of these interneurones A preliminary report has been given (Jankowska *et al.* 1965)

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Fig 1 I terneurone in which lat longlasting actions are evoked from FRA after administration of DOPA. Upper traces are extracellular microelectrode recordings in A—P and the dorsal root potential from the most caudal L6 rootlet in Q. Lower traces in this and all the other figures are from the L7 dorsal root entry zone. Records A—F taken before an injection of DOPA, show irregular spontaneously ppearing bursts of discharges (A—C) and no clear response to short train of stimuli in either ipsi- or contralateral nerves (D—F I contrast, after 100 mg/kg of DOPA olleys in i FRA (G, H K, L, N) evoke longlasting excitation of the in erneurone while stimulation of co FRA produces an inhibition. The inhibitory action from FRA is revealed by their effects on test discharges evoked from the G-S nerv (M—P) The latter discharge is completely (O) or partially (P) inhibited depending on the interv l between conditioning (cond) and testing olleys. Stimulus strengths are given i multiples of threshold trengths Drawing t lower left represents transverse section of the spinal cord (L7 lev l with location of the in erneurone illustrated at x.

Methods

All experiments were made on unanaesthetized acute spinal cats. For general experimental procedures and drugs employed see Andén *et al.* (1966a) Records were obtained from interneurones as described by Hongo *et al.* (1966) only extracellular records being made; the microelectrodes were filled with 3M KCl solution. The procedure for determining the location of the interneurones was also described by Hongo *et al.* (1966) In all experiments both ventrolateral funicles were dissected in the lower thoracic region and mounted for stimulation in the descending direction. All the records of neurone activity reported here were from interneurones, i.e. neurones which could not be activated antidromically from either the lower thoracic region or the ventral roots (L7 and S1) For abbreviations see Jankowska *et al.* (1967)

Results

I. *Types of interneurones*

Exploration of the spinal cord with microelectrodes showed that after injection of DOPA (100 mg/kg) interneurones activated after a long latency by volleys in the FRA are found mainly in the dorsolateral part of Rexed's layer VII (Rexed 1954) Fig. 1—3 illustrate the discharge of different types of interneurone in this region, in each case after injection of DOPA.

Type A. The discharge of the interneurone illustrated in Fig 1 is evoked from ipsilateral high threshold joint and muscle afferents (G H, K, L, N) but there is no corresponding discharge evoked from the contralateral side (I and M) Volleys in cutaneous afferents were also effective in producing the late discharges, but volleys in group I (J) muscle afferents never did. In some cells volleys in group II muscle afferents produced a discharge (K) but for many of the cells volleys in group III

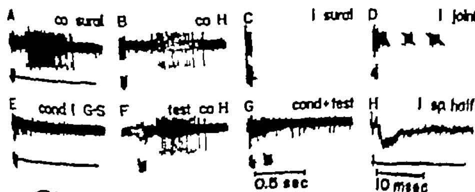


Fig 2. Interneurone activated from the co FRA Extracellular recording after injection of 100 mg/kg of DOPA. A, B, late actions from the co FRA; C—E, lack of response from the i FRA. (Inhibitory effect of stimulation of the ipsilateral high threshold muscle afferents on test discharges evoked from co FRA. H shows the effect of stimulation of the ipsilateral spinal half (i sp. half) at lower thoracic level. Note different time calibrations for A—G and H. Other indications as in Fig

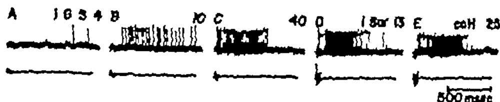


Fig. 3. Interneurone activated from both the ipsi- and contralateral FRA. Records A—C show the effect of increasing strength of stimulation of the l G-S nerve. Other indications as in Fig. 1. Preparation injected with 100 mg/kg DOPA.

afferents were required and when group II volleys gave an effect there was always an increase in the duration of the discharge when stimulation was increased to activate the higher threshold group III afferents. Volleys in high threshold afferents from flexor and extensor muscles were equally effective in producing these late discharges. *Fig. 1 M—P* shows the effect of combined stimulation of the contralateral and ipsilateral FRA. The conditioning volleys from the contralateral side by itself (M) causes no discharge but very effectively inhibits the test discharge from the ipsilateral side (O); the test discharge alone is shown in N. It should be noted that this inhibition occurs at conditioning-testing intervals exceeding 300 msec and hence cannot be caused by crossed PAD in the terminals of the FRA (*cf.* Jankowska *et al.* 1966). A further feature in Fig. 1 is that records were also obtained from this interneurone before the injection of DOPA (A—F). There is an irregular spontaneous discharge but no definite excitatory effects are evoked by ipsilateral or contralateral volleys (D—F); apparently the characteristic late discharge can only be evoked in this interneurone after administration of DOPA and does not occur in the normal acute spinal state.

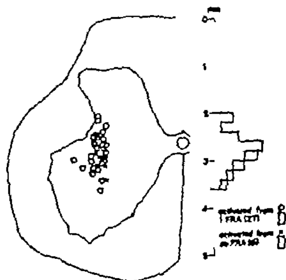


Fig. 4. Location of 27 interneurones with late excitatory actions from i FRA and 6 interneurones with similar actions from co FRA. DOPA (100 mg/kg) had been given. The histogram shows distribution of these interneurones at different depths from the surface of cord dorsum.

Type B Other interneurones in this region of the spinal cord are activated from the FRA of the contralateral but not the ipsilateral nerves (Fig 2) combined stimulation of the FRA of the two hindlimbs revealed inhibition from the ipsilateral nerves of a test discharge evoked from the contralateral side (E—G) This inhibition also occurs at long conditioning-testing intervals and therefore, like the late inhibition seen in Type A interneurones, cannot be caused by PAD in the terminals of the FRA.

Type C. A final type of interneurone received late excitation from the FRA of both the ipsilateral and contralateral nerves (Fig 3)

Of the 47 interneurones sufficiently studied, 31 were activated only from the ipsilateral FRA, 12 only from the contralateral FRA and 4 were activated by the FRA of both hindlimbs. Fig 4 shows the location of the interneurones activated from either the ipsilateral or the contralateral limb there is a slight tendency for the latter type to lie more ventrally In several of the experiments multi-unit recordings were made which were not suitable for analysis of the reciprocal actions from the two hindlimbs, but which provided good localization of the interneurones. It is our impression from these multi unit recordings that the great majority of these interneurones are located at a depth of 2.6—2.7 mm from the cord dorsum in the dorsolateral part of Rexed's (1954) layer VII and the scattering in Fig 4 may not be entirely representative. Recordings made on many occasions from slightly more medial regions never gave any discharges of either single or multi-unit type.

In section 3 below it will be shown that many of the interneurones with a late longlasting discharge may also be activated from the FRA after a short latency

2. *Inhibition of the late discharge by longlasting stimulation*

The late effects evoked after DOPA in primary afferents and motoneurones can be inhibited by volleys in the same primary afferents from which these effects are evoked (Andén *et al.* 1966b) Hence if the interneurones described in the last section are engaged in mediating these actions a similar phenomenon would be expected. The interneurone in Fig 5 receives the typical longlasting excitation from the ipsilateral FRA and these discharges could be inhibited by volleys in the contralateral FRA. The records in Fig 5 show that when the repetitive stimulation is prolonged the onset of the discharge occurs after a progressively longer latency Hence it can be concluded that transmission to these interneurones can be inhibited as outlined above.

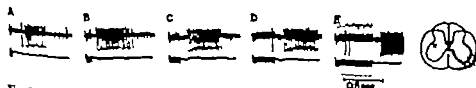


Fig. 5. Interneurone showing delay in the onset of the late discharge with prolongation of the repetitive stimulation of the ipsilateral sural nerve. Recording was made after injection of 100 mg/kg DOPA. Other indications as in Fig. 1

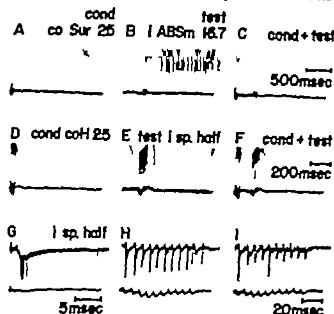


Fig. 6. Inhibitory effects from the co FRA of the descending monosynaptic transmission to an interneurone activated from the i FRA. DOPA (100 mg/kg) had been given. Part of the records in E and F are shown in H and I respectively which were obtained at faster sweep speed. Repetitive stimulation and single sweep in all records except in G where single shock stimulation and superimposed sweeps were applied. Conditioning volleys in the co FRA (alone in A) inhibit (C) the test discharge evoked from the i FRA (alone in B). E, G and H show the monosynaptic activation by descending volleys in the ipsilateral ventrolateral funicle (i sp. half). By conditioning volleys in the co FRA (D) this discharge is inhibited (F, I).

3. *Other excitatory effects*

In all experiments the effect of stimulation of the ipsilateral and contralateral spinal cord in the lower thoracic region was tested in order to exclude that any of the cells belong to ascending pathways. A descending volley in the ipsilateral spinal cord gives rise to a large monosynaptic focal potential in the region where the interneurones are located (Fig. 2 H). Correspondingly many of these cells receive a monosynaptic excitatory action, as illustrated in Fig. 6, G for an interneurone activated from the ipsilateral FRA and inhibited from the contralateral FRA. Fig. 6, E and

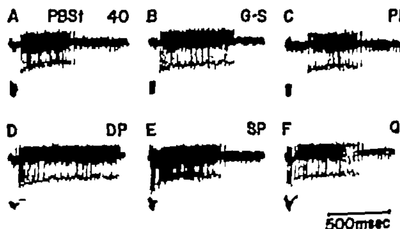


Fig. 7. Interneurone with late longlasting discharge from the FRA which in addition is activated from some nerves after a shorter latency (E, F). Recording was made after injection of 100 mg/kg DOPA.

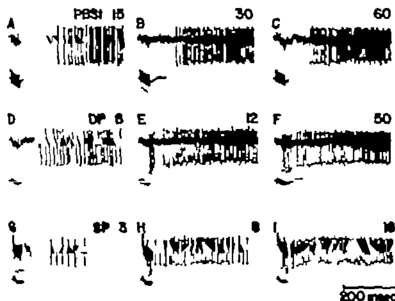


Fig. 8. The late and early discharges evoked at increasing strength of stimulation. Note an increase in frequency of the late discharge and prolongation of the duration (G—I) with increasing strengths of stimulation but also appearance of the early discharge and shortening of the delay in onset of the late discharge. Recording was made after injection of 100 mg/kg DOPA.

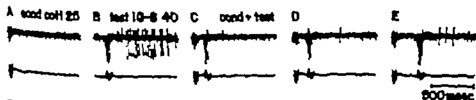


Fig. 9. Inhibition of the late and early discharges from the i FRA by volleys in the co FRA. The average number of early spikes evoked from i FRA (B) was 4 and when conditioned by volleys in co FRA (A) it was 3 in C, 3 in D and 4 in E. The stronger inhibitory effects on the late than on the early discharge might suggest that contralateral inhibition in this case was exerted mainly on the pathway transmitting the late discharge to the interneurones whose activity was recorded. DOPA (100 mg/kg) had been given.

F with the corresponding faster records H and I show that this monosynaptically evoked discharge can also be inhibited after a long interval by conditioning volleys in the FRA of the contralateral limb, but not as effectively as the late discharge evoked from the ipsilateral FRA.

Many of the interneurones which receive the late longlasting activation from the FRA are also excited after a short latency from the same afferents (Figs. 7—9). In the interneurone of Fig. 7 the typical late discharge is evoked from all nerves but in addition there is a short latency discharge from SP (E) and Q (F) nerves. The latency of the early discharge varies from 6—25 msec (from the first incoming group I volley in the train). In some interneurones the early discharge was evoked from all the nerves tested but in others only from some of the nerves and then usually from those nerves which are anatomically large. Usually the early discharge could be

evoked both from high threshold muscle afferents and from cutaneous afferents. In the great majority of interneurones there is an interval between the end of the early discharge and the onset of the later. However, with strong stimulation of cutaneous nerves tending to prolong the early discharge and shorten the latency of the late one, these discharges may be almost continuous (Fig. 8 H and I). Attempts to investigate whether the early discharge remains after recovery from the effects of DOPA were not successful and we are therefore unable to state if the early discharges occur in these cells in the acute spinal cat not injected with DOPA. Fig. 8 illustrates that the early discharge occurs at somewhat higher stimulation strength than the late discharge. The effect of conditioning volleys from the contralateral limb on the early and late discharges is shown in Fig. 9. Transmissions of both actions are inhibited, but the latter much more effectively. During the complete inhibition of the late discharge in C and D the number of spikes in the early discharge is reduced from 4 to 3, but at a longer interval (E), when there is still effective inhibition of the late discharge, there is no decrease in the number of spikes of the early discharge.

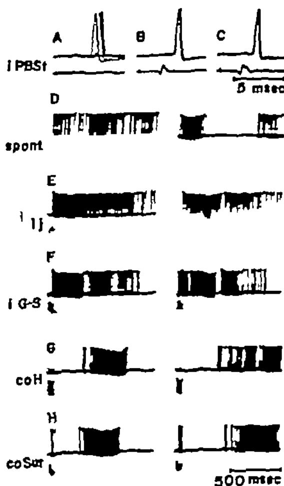


Fig. 10. Monosynaptic discharge evoked from group I afferents (A—C) in an interneurone with longlasting discharge from the i FRA (E, F). Double records are shown for each kind of peripheral stimulation because of the variability of the responses and fluctuation in the spontaneous activity (D) of the interneurone. In records G and H co FRA are stimulated. Recording was made after injection of 5 mg/kg of Nialamide and 50 mg/kg of DOPA.

In 3 interneurones out of 80 there was monosynaptic excitation from group I muscle afferents. One of these interneurones is illustrated in Fig 10 with the monosynaptic activation shown in A—C these records clearly suggest an action from Ia afferents. Records D—H are shown with two traces in each record to illustrate the great variability Nialamide had been given to the cat prior to the administration of DOPA and the spontaneous discharges as well as the variability in evoked discharges are typical in these conditions (*cf* below) The fact that during the early part of the traces a discharge is evoked from the ipsilateral FRA and a pause from the contralateral suggests that this interneurone should be classified as one receiving ipsilateral excitation and contralateral inhibition. However later in the trace there is simultaneous activation from both hindlimbs, which makes a definite classification impossible. The other interneurones with monosynaptic excitation from group I afferents were also difficult to classify since in each case Nialamide had been given.

4. *Correlation with events in motoneurones*

Further evidence that the interneurones with the late longlasting discharge are engaged in transmitting effects to motoneurones has been obtained in experiments

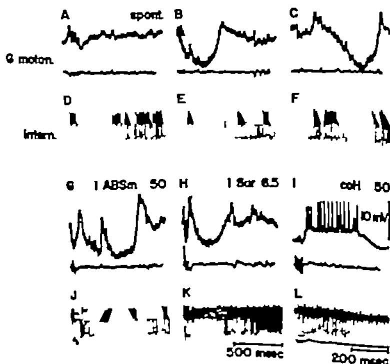


Fig 11 Comparison of spontaneous activity and of responses evoked by volleys in I and co FRA in an extensor motoneurone (A—C, G—I intracellular records) and in an interneurone (D—F, J—L, extracellular records Recording after administration of 10 mg/kg of Nialamide and 1X mg/kg of DOPA. Intracellular recording with DC amplifier voltage calibration in I

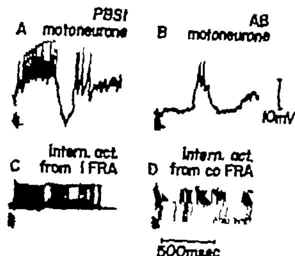


Fig. 12 Comparison of effects from the FRA on two motoneurones (A, flexor and B, extensor) and two interneurones (C, activated from the i FRA as in Fig. 1 and D, activated from the co FRA as in Fig. 2). The i FRA were stimulated in all records. Recording was made after injection of 50 mg/kg DOPA after pretreatment with 5 mg/kg Nialamide. See text.

in which DOPA was given after pretreatment with the monoamine oxidase inhibitor Nialamide. Under these circumstances spontaneous fluctuations of the membrane potential occur in the motoneurones (Jankowska et al. 1967). Correspondingly there are also spontaneous bursts of discharges in the interneurones in which the late discharges are evoked after DOPA. This is illustrated in Fig. 11 with records from a gastrocnemius motoneurone in A—C and from an interneurone in D—I. After pretreatment with Nialamide waves of EPSPs are sometimes evoked from the FRA after DOPA. In the gastrocnemius motoneurone of Fig. 11 the volley in contralateral high threshold muscle afferents evoked a late EPSP (I). Volleys in the ipsilateral FRA give waves of EPSPs and similar events also occur in the interneuronal records (J and K).

The records in Fig. 12 were again obtained after pretreatment with Nialamide all the records being taken within a period of 30 min. A shows the EPSP in a flexor motoneurone and C below an interneurone activated from the ipsilateral FRA. After about 350 msec there is a removal of the EPSP followed by its re-establishment. Correspondingly there is also deceleration of the discharge in the interneurone (C). B shows that during this period a reciprocal EPSP is evoked in the extensor motoneurone and a burst of impulses (D) in an interneurone which was of the type that received its main late activation from the contralateral FRA. The very good correspondence between the establishment and the removal of the EPSPs in the motoneurones and the interneuronal discharges give strong support for the view that these interneurones are concerned with transmission to motoneurones.

5 *After-discharge in other types of interneurones*

Many interneurones in the dorsal horn and intermediary region are activated by volleys in the FRA (Eccles et al. 1956, Eccles et al. 1960, Hongo et al. 1966). Single

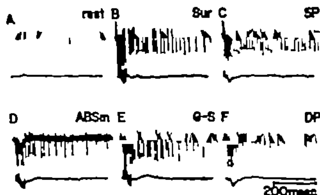


Fig. 13 After-discharge evoked in an interneurone by short train of volleys in the FRA. A shows resting discharge, B—F discharge evoked from the FRA. Cat not injected with DOPA.

volleys in the FRA usually evoke a repetitive discharge of impulses and a short train of volleys in the FRA produces an intense discharge, which in many interneurones is followed by an after-discharge which may last for one sec or more. Interneurones of this type are also found in more ventral regions in the gray matter The after-discharge may be continuous with th initial discharge (Fig 13 C—F) but if the initial discharge is particularly intense there may be a short interval before the onset of the after-discharge (Fig. 13 B) It seems likely that these after-discharges explain the longlasting tail of synaptic action often evoked in motoneurones by a train of volleys in the FRA in the unanaesthetized spinal cat (Jankowska *et al.* 1967 Fig 5) Recordings from many interneurones in th dorsal horn and intermediary region before and after DOPA have failed to indicate an increase in duration of the after-discharge or a change in its temporal characteristics so as to become of a more recruiting type after an injection of DOPA. On the contrary it is our impression that the after-discharges in th interneurones n the dorsal horn, in the intermediary region and also in other parts of the gray matter have a shorter duration after DOPA. However to be certain about this t is necessary to record from the same interneurone before and after the injection of DOPA, because the after-discharges vary enormously from cell to cell.

Discussion

The reflex actions from the FRA n an acut spinal cat injected with DOPA differ in very characteristic fashion from those found in the normal acut spinal cat (*cf* Andén *et al* 1966 b Jankowska *et al* 1967) Among the features that guided us in the search for the nterneurones, which may transmit the reflex actions after DOPA, were not only the long latency duration and characteristic recruiting character of these effects, but also that transmission could be inhibited by volleys in the same afferent from which they re evoked. Furthermore interneurones transmitting effects to different neuronal systems would have characteristic differences in the receptive field from which excitation is drawn. I terneurones transmitting the lat PAD

afferent terminals would be expected to be activated from a bilateral receptive field, interneurones transmitting the late EPSP to flexor motoneurones from an ipsilateral receptive field, and the corresponding interneurones transmitting excitation to extensor motoneurones should receive excitation from a contralateral field By all these criteria the present report identifies the interneurones transmitting the late longlasting actions to primary afferents and to motoneurones after DOPA. They are located in a fairly limited region in the dorsolateral part of Rexed's (1954) layer VII dorsal to the motoneuronal nucleus. No corresponding late discharges were found in interneurones in the dorsal horn and intermediary region.

The interneurones, which receive excitation from either one or other hindlimb are of particular interest. The latency and duration of the discharges and the fact that they are not evoked without DOPA in the acute spinal cat (Fig 1) virtually suffice to identify these interneurones with those transmitting effects to flexors and extensors respectively and further strong support for this identification is provided by the finding that transmission to them can be very effectively inhibited from the hindlimb opposite to that providing the excitation. Such a reciprocal organization of effects from the two hindlimbs was postulated on the basis of the analysis of transmission to motoneurones and it is clear that the present recordings from interneurones strongly support the scheme given by Jankowska et al (1967) in their Fig 17 Very strong evidence is thus provided for the *half-centre organization of the interneurones transmitting the effects to motoneurones after DOPA*

The extremely long duration of the effects from the FRA after DOPA deserves special discussion and should also be considered in relation to the long latency after which the effect may occur With the interneuronal recording discharges lasting well over a second have been observed and records from motoneurones suggest that the interneuronal discharges evoked from the FRA may last 30 sec or more These prolonged discharges are of general interest in relation to the problem how spinal reflexes can contribute to movements. Most natural movements are slow processes caused by sustained motoneuronal discharge By contrast most reflex actions previously studied are of a d emblée type (cf Creed et al 1932) and would require a maintained afferent discharge in order to produce a motoneuronal discharge of some duration. However with many reflexes the reflex movements may unload the receptor and decrease the afferent discharge. Reflex actions, which can be sustained because of a long duration of intraspinal processes, seem therefore well suited to subserve natural movements. The present results do not give any information regarding the mechanism by which these longlasting discharges are produced. The most obvious possibility is a positive feed-back involving transmission in a closed chain of interneurones (cf Lorente de Nó 1938) However we cannot exclude the alternative possibility that the longlasting discharges may be generated in an open chain, either by an exceptionally longlasting synaptic transmitter action, or by a delayed depolarization of the type, which under certain conditions may follow a single spike in the stretch receptor neurone in the lobster and which may evoke several hundred impulses (Grampp 1966)

The long latency of the discharge is a more puzzling feature than the long duration. It is hardly likely that latencies of 0.1 to 0.5 sec are caused by transmission through a large number of interneurones each activated after a short delay. It is more probable that transmission occurs through an interneuronal chain with a relatively small number of cells, some of which may require considerable temporal summation for their activation. It is, however, of considerable interest that in many interneurones activation occurs in two phases. There is an early discharge after a relatively short latency which after an interval is followed by the late, longlasting discharge (Fig. 7—9). The early discharge permits a classification in three main types. 1) Cells with early and late discharges from all nerves. 2) Cells with late discharges from all nerves but early discharges only from a few of these nerves. 3) Cells without early discharges but with late discharges from all nerves. Various circuit diagrams have been devised to explain these three types of discharge but it would serve little purpose to present these diagrams now since only further experiments can show their value. In any case it is likely that the existence of the early discharge will be a factor of great importance in future attempts to analyse the processes responsible for the long duration of the discharge in these interneurones.

Examples of other problems that require a further experimental analysis are: Where is the inhibition exerted that is produced by impulses in the afferents from which the late discharge is evoked? Is the early discharge as the late one released after DOPA, or is it evoked also before the injection of DOPA? What is the significance of the monosynaptic discharge evoked from Ia afferents in some of the interneurones? What is the function of the descending monosynaptic connections; can alternating activity in the half-centres be evoked also by descending impulses? However, the main problem is the mechanism for the inhibitory action that constitutes such an essential element in the half-centre organisation, i.e. the mutual inhibitory interaction between the interneuronal paths transmitting excitation to flexor and extensor motoneurones. In this connection it is of interest that the early discharge is less effectively inhibited from the other limb than the late (Fig. 9) despite the fact that the latter action seems to have a higher safety factor of transmission (Fig. 8). These findings suggest that the late discharge is inhibited at some critical site.

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The Effect of DOPA on the Spinal Cord

7 Reflex activation of static γ-motoneurones from the flexor reflex afferents

By

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Abstract

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1 unanaesthetized spinal cats reflex activation of γ-motoneurones (to flexor muscle) from the flexor reflex afferents (FRA) has been assessed from the discharge recorded in muscle spindle afferents. After injection of L-DOPA the resting discharge frequency increases i fferents with secondary endings and volleys in the FRA evoke longlasting acceleration of the discharge in these afferents On the basis of previous experiments showing that spindle afferents with secondary endings are activated from static but not from dynamic γ-motoneurones it is inferred that static γ-motoneurones are spontaneously acti and reflexly activated from the FRA after an injection of DOPA. I has been confirmed that in the acut spinal cat, not treated with DOPA, there is no or little evidence for reflex activation of static γ-motoneurones from the FRA and it is postulated that the reflex pathway to static γ-motoneurones is inhibited in this preparation. The results are discussed in relation other reflex effects evoked from the FRA

The spinal reflexes produced from the FRA (flexor reflex afferents) after an intravenous injection of DOPA are characterized by a late longlasting discharge in α- and γ-motoneurones and a late longlasting primary afferent depolarization (Andén *et al* 1966a) Th effects to α-motoneurones and primary afferent terminals ha e been analyzed prev ously (Andén *et al* 1966b Jankowska *et al* 1967a, b) The present paper deals with some aspects on the late reflex discharge in γ-motoneurones Many investigators ha e demonstrated that γ-motoneurones are co-activated with α-motoneurones n certain pinal reflexes (Hunt 1951 Kobayashi *et al.* 1952, Eldred and Hagbarth 1954 Hunt and Paintal 1958, Voorhoeve and van Kanten 1962) With the recent demonstration of dynamic and static γ-motoneurones (Matthews 1964 Bessou and Laporte 1966) it is necessary to consider that there may be differences in reflex connections to these types. From experiments with recording from muscle

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spindle afferents Alnaes *et al.* (1965) have postulated that dynamic γ-motoneurones participate in spinal reflexes but there was no evidence that static γ-motoneurones were involved. Appelberg *et al.* (1966) have shown that the sensitivity of secondary endings is increased from static but not from dynamic γ-efferents. In the present investigation recording from afferents with secondary endings has shown that static γ-motoneurones are reflexly activated from the FRA after an injection of DOPA. Some of the results have been discussed in a review (Lundberg 1966)

Methods

All experiments were made on unanaesthetized low spinal cats which were decerebrated by intercollicular transection. Both hindlimbs were denervated except for the left tenuissimus muscle. The nerve to this muscle was dissected free and placed on electrodes but was in intact connection with the muscle. The tenuissimus muscle was severed at the level of the knee joint and the central end connected to a strain gauge. The discharge in spindle afferents from tenuissimus was recorded from thin dorsal root filaments (L7). The afferents were classified according to their conduction velocity and response during stretch and contraction. A number of nerves in the left hindlimb were dissected for stimulation. Short trains of stimuli were given to evoke the reflex effects. For other experimental procedures and drugs, cf. Andén *et al.* (1966a).

The following abbreviations are used: anterior biceps-semimembranosus, ABSm; posterior biceps-semitendinosus, PBSt; gastrocnemius-soleus, G-S; tenuissimus, Ten; suralis, Sur; flexor reflex afferents, FRA; primary afferent depolarization, PAD.

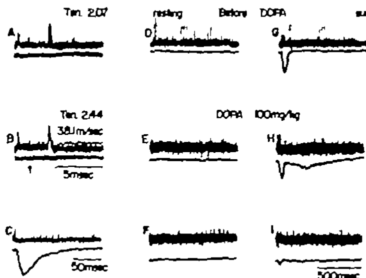


Fig. 1. Reflex activation from cutaneous afferents of a muscle spindle afferent with secondary ending. Upper traces are recordings from an afferent in a thin dorsal root filament of L7. The tenuissimus nerve is stimulated at threshold for the axon in A and at a strength slightly above threshold in B. Stimulus strengths are given as multiples of threshold strengths and the conduction velocity of the axon is given in B. Lower traces in A and B are from the L7 dorsal root entry zone. The arrow in B indicates the time of arrival of group I volley from the tenuissimus nerve. In C the lower traces record tension in the muscle. In C there is a pause during the twitch evoked by a single volley in the nerve to tenuissimus. In the middle column D–F the resting discharges and in the right (G–I) the effects of a short train of volleys in the ipsilateral sural nerve. D and G were taken before, E, F, H and I 10 min after [illegible] intravenous injection of DOPA, 100 mg/kg. After DOPA there is an increase in the resting discharge (E) and a long lasting acceleration is evoked by stimulation of the sural nerve (H). In the lower records F and I the resting tension in the tenuissimus muscle is decreased; the frequency of the resting discharge is lower but a considerable acceleration [illegible] increased [illegible]. I. Superimposed traces in A and B, single traces C–I.

Results

The discharges in Fig 1 are from a spindle afferent with a secondary ending A and B are shown for identification there is threshold stimulation for the afferent in A at 2.07 times the threshold of group I fibres and suprathreshold stimulation in B The arrow indicates the arrival of the group I volley in the recording from the dorsal root entry zone. The conduction velocity for the afferent, 38 1 m/sec is in the group II range. Stretch of the tenuissimus muscle gave a discharge in the afferent and

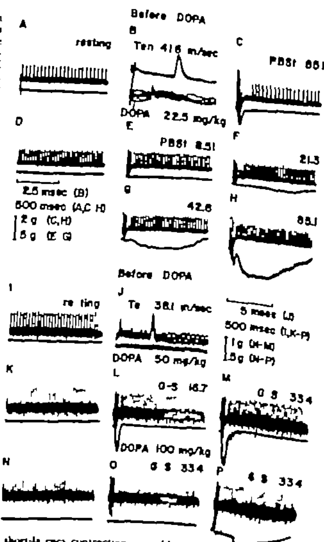


Fig 2 Reflex activation from high threshold muscle afferents to two different muscle spindle afferents with secondary endings Upper traces in A—H and I—P are records from thin dorsal root filaments of L7 The tenuissimus nerve was stimulated at strength above threshold for the different axons in B and J respectively the conduction velocities are given The tension in the muscle is recorded in the lower traces in all records except B and J which are from the L7 dorsal root entry zone The left column shows the resting discharges An increased resting frequency is seen after injection of DOPA In the first series (A—H) stimulation of the nerves posterior biceps and semitendinosus (PBSt) at strength of 85 1 times threshold, before DOPA, gave pause during contraction but no late acceleration. After 22 5 mg/kg DOPA (E—H) the nerve PBSt was stimulated with increasing strength I F there is late contraction in the muscle and the same time deceleration of the afferent discharge I G stronger extrafusal contraction without deceleration, indicating γ-activation compensating the unloading effect of the extrafusal activity I H increased extrafusal contraction accompanies an acceleration of the afferent After injection of 50 mg/kg DOPA the second series (I—P) there is long-lasting acceleration after stimulation of the nerve gastrocnemius-soleus G-S though the short-latency contraction is blocked After ... of DOPA there is block of the early extrafusal contraction and an appearance of the late ... O here ... long-lasting acceleration ... thout ... nd in P ... h ext afusal ... traces in B and J single traces ... all others

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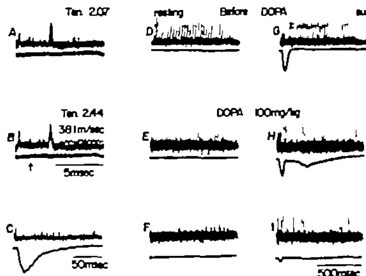


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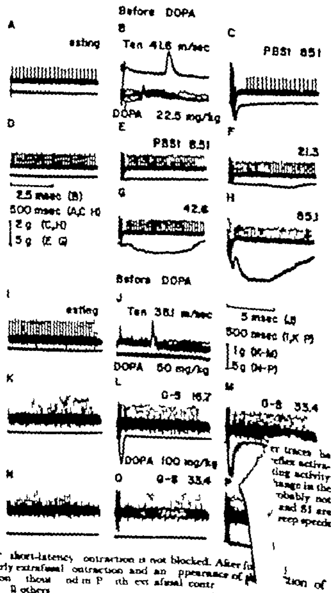


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there was no apparent dynamic sensitivity Record C shows the pause during contraction of the muscle evoked by stimulation of the tenuissimus nerve before DOPA. Records D and G were taken before DOPA. G shows the effect of a train of volleys in the sural nerve with a reflex contraction in the muscle, and during this period there is a pause in the afferent discharge. Afterwards the discharge rate is resumed with no evidence of a late acceleration. Records E, F H and I were taken after DOPA. The resting length of the muscle has not been changed but there is a clear increase in frequency of the resting discharge from 33/sec in D to 42/sec in E (cf also Fig 2) Stimulation of the sural nerve (H) now gives a smaller early and a late reflex contraction, both being expected from the efferent reflex discharge evoked after DOPA (Andén et al. 1966a) At the same time a clear-cut longlasting acceleration of the afferent discharge is found This is even more clearly visible in the lower records F and I which were obtained at shorter resting length The resting discharge i now less than th initial one before DOPA but nevertheless the acceleration following stimulation of the sural nerve is very marked.

Fig 2 illustrates similar effects evoked from high threshold muscl afferents. In both group II afferents illustrated in this figure there is a late reflex acceleration after DOPA but there is some difference In the afferent from which I–P were taken there is a marked late acceleration after a small dose of DOPA n the absence of any late extrafusal contraction After more DOPA had been injected th acceleration could be evoked by volleys that neither evoked an early nor a late extrafusal reflex contraction Hence there is evidence that in the late reflex evoked after DOPA γ-motoneurones may be activated at lower threshold than α motoneurones (cf Hunt 1951) In the afferent from which A—H were taken the frequency during the reflexly evoked extrafusal contraction G is almost the same as n the resting record (D) and an acceleration is produced only with the stronger afferent stimulation in H Presumably the unloading effect of the extrafusal contraction in C is compensated by an increased γ-discharge F shows that a deceleration of fferent discharges may occur during the extrafusal late reflex contraction. Records D H are taken to indicate parallel activation of α and γ-motoneurones in the late reflex

Fig 3 illustrates that there is a similar discharge evoked in Ia afferents large spike) In this experiment the L7 and S1 ventral roots were transected and the reflex activation occurs through a few efferent fibres in the L6 ventral root

The sensitivity of muscle spindle receptors is influenced by noradrenaline Hunt 1960) and it was necessary to investigate if DOPA could exert it ction at a peripheral level. The control experiment in Fig 4 excludes this. The ventral roots L6—S1 were severed before DOPA was given and stimulation of the muscle efferents is made in the ventral root at a strength chosen to be supramaximal for α- and γ-efferents. There is no late acceleration in the discharge rate after DOPA Control experiments also exclude that the discharges are caused by activity n th sympathetic nervous system because after transection of all the efferents to the tenuissimus no late afferent acceleration of the discharge was found after supramaximal stimulation of cutaneous or muscle afferents. It can therefore be concluded that the acceleration is evoked by

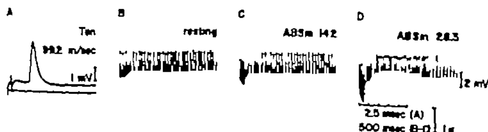


Fig. 3. Reflex activation from the FRA after DOPA of a muscle spindle afferent with primary ending. Upper traces in A—D are from a dorsal root filament of L7. The tenuissimus nerve is stimulated at a strength above threshold for the axon in A, and the conduction velocity of the axon is given. Note different amplification and sweep speeds in A and B—D. The large spike in B—D corresponds to that in A. Lower traces record tension in the muscle, sensitivity indicated. Ventral roots L7 and S1 were cut leaving only a small part of the α- and γ-innervation in L6 intact. Resting activity after administration of 70 mg/kg DOPA is recorded in B. In C and D the nerve from anterior biceps and semimembranosus (ABSm) is stimulated with a short train of pulses at different strength. In D there is a reflex activation of the spindle afferent, which coincides with the late contraction in the tenuissimus muscle. Superimposed traces in A, single in B—D

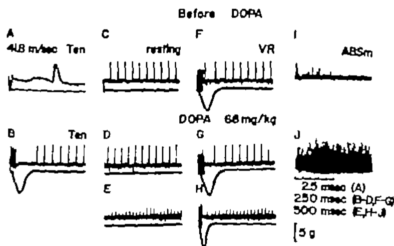


Fig. 4. Control experiment on de-efferented muscle. Upper traces in A—H, recorded in a thin dorsal root filament, are from a tenuissimus afferent with secondary ending. The spike in A was evoked from the tenuissimus nerve and the conduction velocity of the axon is given. Stretch of the tenuissimus muscle evoked a discharge which paused during contraction of the muscle (B). The ventral roots L6—S1 were transected, the central end of L7 was mounted for recording, the peripheral end of L7 and S1 for stimulation. Superimposed traces in A, single in B—J. In the lower traces the tension in the muscle is recorded (sensitivity as indicated). I and J are records of the reflex activation from ABSm in a ventral root filament before (I) and after (J) DOPA. The resting activity before (C) and after administration of 66 mg/kg DOPA is recorded in C—E. There is a change in the resting frequency from 20/sec before to 22/sec after DOPA. This slight change is probably not significant. In F—H the effects of supramaximal stimulation of the ventral roots of L7 and S1 are recorded. There is no change in the response to stimulation after DOPA. Note different sweep speeds in E and H. Superimposed traces in A, single in B—J

the late reflex discharge that is evoked in γ-motoneurones after an injection of DOPA.

Discussion

There is now much evidence that supraspinal centres can exert a differential control of the dynamic and static sensitivity of the muscle spindle (Jansen and Matthews 1962, Appelberg 1962, Appelberg and Emonet Dénand 1965). In fact the experimental analysis showing the existence of dynamic and static γ-motoneurones originated from observations on the supraspinal control of this sensitivity. Other experiments by Alnaes *et al.* (1965) have revealed that a differential control also exists at the spinal reflex level. In spinal cats stimulation of nerve trunks will reflexly change the sensitivity of the muscle spindle in a manner that would be expected if there was a reflex activation of dynamic but not of static γ-motoneurones. In view of the differences in reflex effects evoked from the FRA before and after DOPA (Andén *et al.* 1966a, b 1967) it was of obvious interest to investigate if static γ-motoneurones are activated from the FRA after DOPA.

It has been a consistent finding that the discharge in afferents with secondary endings increases in frequency during the late reflex effect after an injection of DOPA. Our control experiments exclude that the effect is peripheral or mediated via the sympathetic nervous system and on the basis of the findings by Appelberg *et al.* (1966) it is therefore postulated that static γ-motoneurones are reflexly activated from the FRA after an injection of DOPA. In confirmation of Alnaes *et al.* (1965) it was found that there is little or no evidence for reflex activation of static γ-motoneurones in spinal cats not injected with DOPA. It is therefore postulated that the reflex path to static γ-motoneurones is inhibited in this preparation. This is analogous to the tonic inhibition of the pathway from the FRA to I afferent terminals (Andén *et al.* 1966b). So far our experiments have only been concerned with the ipsilateral flexor reflex. For the further discussion it will be assumed that static γ-motoneurones are also activated in the late crossed extensor reflex evoked after an injection of DOPA (Jankowska *et al.* 1967). Our results indicate that in this reflex there is a close linkage in the activation of α motoneurones and static γ-motoneurones but some evidence was obtained suggesting that the threshold relationship of these two components of the reflex is not absolutely fixed. From Fig. 2 and from the records by Jankowska *et al.* (196 a) it can be concluded that the reflex discharge in ipsilateral γ-motoneurones to flexors can be inhibited by volleys in the contralateral FRA in the same way as was found for α motoneurones. In analogy with the findings regarding connections to α motoneurones it is therefore suggested that also the reflexes to γ-motoneurones from the two hindlimbs are reciprocally organized with inhibition exerted at an interneuronal level (*cf.* Jankowska *et al.* 1967a, b).

After DOPA there is an increase in the resting discharge from secondary endings. Since there is no corresponding effect in the de-efferented muscle the most likely explanation is an increased resting discharge in static γ-motoneurones. However when comparing efferent discharges in the tenuissimus nerve before and after DOPA Andén *et al.* (1966a) and Jankowska *et al.* (19 a) found no significant change of the frequency in the whole resting γ-discharge. The present result therefore raises the question whether an increase in resting discharge in static γ-motoneurones is

Fig. 5. Diagrammatic representation of pathways giving the actions from the FRA found before and after DOPA in the acute spinal cat. In the left diagram are shown the paths giving the following three effects from the FRA found in the normal acute spinal cat: the negative feed-back represented by the PAD in the FRA, inhibition of transmission from Ia to Ia afferents, activation of dynamic γ-motoneurones. The right diagram shows the pathways giving the actions found after DOPA. After inhibition of the pathways in the left diagram there is now PAD from the FRA in Ia afferents and activation of static γ-motoneurones.

accompanied by a decrease of the resting frequency in dynamic γ-motoneurones. Recordings from single γ-efferents are required in order to solve this problem. It would be expected that the reflex activation of static γ-motoneurones enhances the discharge also in spindle afferents with primary endings. This has been found, but it cannot be concluded from our results that this effect is caused entirely by reflex activation of static γ-motoneurones. The present experiment gives information only about the participation of static γ-motoneurones in the late reflex discharge and it is not known whether or not the dynamic ones are activated.

Even without this knowledge it may be appropriate to summarize some of the effects regarding reflexes before and after DOPA in relation to the finding that static γ-motoneurones are engaged in the late reflex response. The left diagram in Fig. 5 shows some of the connections in the acute spinal cat not injected with DOPA. There is short-latency activation from the FRA to dynamic γ-motoneurones. Through this reflex there will be an increased dynamic sensitivity in Ia afferents but no discharge evoked in group II afferents. There is a negative feed-back to the FRA (Eccles *et al.* 1962) giving presynaptic inhibition of the transmission from these afferents (Lund *et al.* 1965). This action will tend to decrease the central effect of impulses in group II afferents evoked independently of the γ-bias. The diagram also shows an inhibitory interactive connection described by Lund *et al.* (1965) through which transmission from Ia afferents of flexors to Ia afferent terminals will be inhibited at an interneuronal level. Through this inhibitory interactive mechanism there can occur a removal of a background PAD which may exist in Ia afferent terminals, and hence facilitation of transmission from Ia afferents. To summarize: The presynaptic and interactive connections all seem to favour transmission from Ia and depress transmission from group II afferents, thus providing optimal cond[illegible] for the operation of the Ia γ-loop.

The right hand diagram in Fig. 5 shows the connections revealed by [illegible] of DOPA. Short latency transmission from the FRA to dynamic [illegible] depressed and so is the transmission in the negative feed-back pathway [illegible] to the FRA (Andén *et al.* 1966a). There is however a depression [illegible]

inhibiting transmission from Ia to Ia afferents but instead a release of transmission in the pathway *from the FRA to Ia afferent terminals* (Andén *et al.* 1966b). The reflex activation of static γ motoneurones would give impulses in group II afferents *with secondary endings* but also in Ia afferents with primary endings. It is of considerable interest that the absence of a negative feed-back from the FRA now seems to favour transmission from group II afferents, whereas there is presynaptic inhibition of transmission from Ia afferents evoked not only from the FRA (Andén *et al.* 1966b) but also from Ia afferents when the inhibitory interactive mechanism of Lund *et al.* (1965) does not operate. Hence the total pattern of reflex connections is less favourable for transmission from Ia afferents and seems to be laid out for operation of a group II γ-loop.

The concept of a group II γ-loop is tentative and further experiments will have to show if it is useful. The only known reflex effects from group II afferents are those caused by the probable participation of these afferents among the FRA (Eccles and Lundberg 1959, Holmqvist and Lundberg 1961, Lundberg 1966). The scheme in Fig. 5 may get a completely different significance if other reflex action from afferents with secondary endings are discovered. At present it is only possible to forward the suggestion that group II discharges may contribute in maintaining the longlasting activation of α-motoneurones. Jankowska *et al.* (1967a) postulated that an intraspinal positive feed back in the pathway from the FRA to motoneurones is an essential mechanism in the production of the longlasting discharges after DOPA. External γ loops may also contribute in maintaining these discharges. The reflex activation of static γ-motoneurones from the FRA may give further activation of α-motoneurones via group II afferents and this effect may be enhanced by the possible co-activation of static γ-motoneurones giving an external positive feed back loop. The γ-discharge also evokes a longlasting discharge in Ia afferents, which may bring about added excitation of α-motoneurones. The latter effect would be counteracted by the presynaptic inhibition of transmission from Ia afferents evoked from the FRA after DOPA but it seems unlikely that this inhibitory process would be able to switch off completely transmission from Ia afferents. The presynaptic effect in Ia afferents may be a mechanism for regulation of the contribution from Ia afferents to the longlasting reflex excitation. It remains to be demonstrated if this is a more useful idea than the above suggestion that the presynaptic inhibition of transmission from Ia afferents may be concerned with a switch from a group Ia to a group II γ-loop.

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Uptake and Release of α-Methyl Noradrenaline in Vitro after Reserpine Pretreatment

A Histochemical Study

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Abstract

HAMBERGER, B. and T. MALMFORS. *Uptake and release of α-methyl-noradrenaline in vitro after reserpine pretreatment.* Acta physiol. scand. 1967. 70. 412—418.

The reserpine-resistent uptake and storage of α-methyl-noradrenaline in vitro were investigated in the adrenergic nerves of the rat iris. After reserpine pretreatment an effective accumulation of α-methyl-noradrenaline took place intraneuronally. The amine remained intraneuronally for several hours when incubated in amine-free medium at 37° C. The morphological appearance changed during the incubation in a manner suggesting that part of the amine was bound to an intraneuronal structure, which might be the amine storage granules. Interference with energy generation in the medium, as well as chemical depolarization by increasing potassium ion concentration in the medium, inhibited accumulation and caused a rapid release of α-methyl-noradrenaline. Protriptyline inhibited the uptake of α-methyl-noradrenaline efficiently and also enhanced the disappearance of accumulated amine. Although (+)-amphetamine was less potent in preventing uptake it produced a rapid release of the amine.

Exogenous noradrenaline (NA) can accumulate in adrenergic nerves of reserpine pretreated animals, provided that a monoamine oxidase inhibitor has been given in advance (Hamberger et al. 1964, Carlsson and Waldeck 1965, Malmfors 1965). The distribution of the exogenous amine as revealed in the fluorescence microscope differs from that in an untreated animal (Malmfors 1965). This is, in all probability, due to blockade by reserpine of the ATP-Mg dependent uptake-storage mechanism in the amine storage granules (Carlsson, Hillarp and Waldeck 1963, Carlsson 1965, Euler and Lishajko 1965). In the present study the reserpine-resistent uptake of α-methyl NA, used instead of monoamine oxidase inhibitor and NA, the release of the accumulated amine and the effect of chemical depolarization and of drugs were investigated in vitro by means of the histochemical fluorescence method of Falck and Hillarp (Falck 1962, Falck et al. 1962, Corrodi and Hillarp 1963, 1964, Corrodi and Jonsson 1967).

Material and Methods

Albino rats (Sprague-Dawley 170—200 g) were pretreated with reserpine (5—10 mg/kg, 4 or 12—18 hrs before the experiments) The rats were killed by decapitation under light ether anesthesia, the eyes were removed and the iris taken out for preparation (Malmfors 1965) A small piece of an iris from each animal was stretched and mounted as whole, as control of the reserpine pretreatment. Each iris was then divided into two pieces, and put into ice-cold Krebs-Ringer bicarbonate buffer containing glucose and saturated with 93.5 O_2, 6.5 % CO_2 (Hamberger 1967) The irides were then moved to 50-ml incubation flasks containing 4 5 ml of buffer and the flasks preincubated in metabolic shaker for 15 min, usually at 37 C. In the experiments on accumulation of α-methyl-NA and its prevention, the medium modifications or the drugs tested were present during preincubation, after which α-methyl-NA was added. The tissue was incubated with amine for 30 min, and stretch preparations were then made

In the experiments on disappearance of α-methyl-NA, the tissue was incubated in the standard Krebs-Ringer medium containing α-methyl-NA for 5 or 30 min. The irides were then rinsed in ice-cold medium for about 10 sec, moved to fresh buffer and incubated further for varying times. In these experiments, medium modifications or addition of drugs were made in the last incubation medium. Stretch preparations were then made of the irides.

In some experiments, reserpine-pretreated rats were injected with α-methyl-NA, 0.2 mg/kg, i. After 15 min, the rats were killed, a small piece of an iris was stretch-prepared as uptake control, and pieces of the remaining irides incubated in vitro for varying times without or in the presence of drugs, after which stretch preparations were made.

When studying the effect of exclusion of glucose the irides were incubated for 15 min in medium without glucose before the last incubation. In some experiments, the incubation flasks were continuously gassed with nitrogen atmosphere (93.5 N_2 6.5 % CO_2) (cf Hamberger 1967)

The stretch preparations of the irides were immediately dried over phosphorus pentoxide treated with formaldehyde gas and examined and photographed in the fluorescence microscope (see Malmfors 1965) Controls were made of the in vitro uptake of α-methyl-NA, and these preparations, together with those made directly after killing the rat and one from an untreated rat, were always treated with formaldehyde gas in the same reaction vessel. Two irides were invariably treated identically in each experiment, and one of them judged without previous knowledge of the treatment. At least two separate experiments were made on each type of treatment.

Drugs Reserpine (Serpasil® Ciba) α-methyl-NA (Corbasil® Hoechst Anilin, AB); 2 4-dinitrophenol (p Merck) (+)-amphetamine (sulphate Sigma); protriptyline (Concordin® Merck, Sharp & Dohme)

Results

Uptake and storage of α-methyl NA after reserpine pretreatment When irides from rats treated with reserpine 12—18 hrs before killing were incubated in a Krebs-Ringer bicarbonate buffer with α methyl NA (Table I) the result was a concentration-dependent fluorescence intensity in the adrenergic nerves, whereas control tissue incubated without α-methyl-NA showed no specific fluorescence (cf Hamberger 1967) The restituted fluorescence was distributed in a pattern different from that in irides of untreated rats (see Malmfors 1965) The varicosities were less pronounced, and the nerve terminals had a smooth appearance Furthermore non terminal axons showed strong fluorescence (Fig 1) Irides which had been further incubated in the standard buffer without α-methyl NA at 37° C after restitution of the fluorescence had a completely different appearance In addition to a slight reduction in fluorescence intensity in the adrenergic terminals (see Table II) the fluorescence was now localized mainly to the varicosities, and the non-terminal axons exhibited low fluorescence intensity (Fig 3) The appearance was, in fact, more like that in an untreated rat (Fig 4) These changes could be seen as soon as after incubation for 15 min without α-methyl NA and were marked after 180 min. The same morphological changes were observed when rats were pretreated with reserpine 4 hrs before

TABLE I Fluorescence intensity of adrenergic terminals in irides of reserpine-pretreated rats after incubation with α-methyl-NA for 30 min (Number of experiments within brackets)

α-Methyl-NA concentration μg/ml			
1	(6 × 10 M)	+ + +	(10)
0 1		+ +	(7)
0 01		+	(4)
0 001	(6 × 10⁻ M)	(+)	(3)
—		—	(10)

Slightly increased background fluorescence
Symbols for the average fluorescence intensity of th adrenergic terminals + = trong + + = moderat + = weak — = none I iew of some variation between preparations treated identically certain intermediate grades are also used, by bracketing the last plus sign. The number of plus signs are not quantitatively related, but are comparable t controls in the same experiment.

killing Reserpine in a concentration of 10⁻ M did not influence the restitution and the restituted fluorescence after 180 min but induced generalized yellow fluorescence in the tissue

Effect of interference with energy generation or depolarization with potassium on the uptake and storage of α-methyl NA after reserpine pretreatment (Table II) Restitution of fluorescence with α methyl NA *in vitro* was largely prevented by incubation in media without glucose where no oxygen was present or where dinitrophenol was added see also Hamberger 1967) When the incubation buffer was well oxygenated and contained

TABLE II Fluorescence intensity of adrenergic terminals in irides of reserpine-pretreated rats af er Krebs-Ringer bicarbonate buffer containing glucose (1 8 mg ml) nd satura ed ith (Number of experiments w thin brackets)

Modification of medium	Restitution of fluorescence by in cubation with α-methyl-NA 1 μg ml 30 min 37 C		0 C
No modification	+ +	(10)	0 (5)
N + CO without glucose	—	(2)	
Dinitrophenol 5 × 10⁻⁴M without glucose	—	(3)	
Dinitrophenol 10⁻⁴M without glucose	+	(5)	
50 % exchange of N to K	+	(3	
100 % exchange of N to K	0	(3)	

Non-terminal axons exhibit strong fluorescence

glucose, restituted fluorescence persisted intraneuronally for a considerable time If, however glucose was excluded from the medium, and the medium was saturated with nitrogen instead of oxygen, or if dinitrophenol was added, the fluorescence intensity declined rapidly with time (Table II) Lowering of the incubation temperature by placing the incubation flasks in an ice-bath completely prevented restitution of fluorescence. Incubation for 180 min at 0° C with a buffer saturated with oxygen, and containing glucose or nitrogen without glucose, did not reduce the restored fluorescence. In this case, the nerve terminals retained their smooth appearance and the non terminal axons remained prominent (Fig 2) Dinitrophenol in high concentration only (10^{-} M) induced a reduction in fluorescence intensity after 180 min, even at this low temperature. Both at a high and a low temperature, dinitrophenol caused the fluorescence to disappear earlier in the nerve terminals than in the non-terminal axons.

Depolarization of the axon membrane by exchanging sodium for potassium markedly prevented restitution of fluorescence. When all sodium was exchanged no restitution could be detected. The rate of disappearance of the restituted fluorescence was also strongly increased by exchanging sodium for potassium, and after 60 min incubation, no fluorescence was detectable. At 0° C, the effect was uncertain

Effect of (+)-amphetamine and protriptyline on the uptake and storage of α-methyl NA after reserpine pretreatment. The restitution of fluorescence after incubation *in vitro* with α-methyl-NA (Table III) was strongly inhibited in the presence of (+)-amphetamine and protriptyline (*cf* Hamberger 1967) Protriptyline was more potent than (+)-amphetamine in preventing restitution. The disappearance of restored fluorescence after incubation with the drugs showed that both drugs accelerated the disappearance, but that (+)-amphetamine was definitely more potent.

Incubation in modified media

93.5% O_2 and 6.5 % CO_2 was used as standard medium

Disappearance of fluorescence after incubation in unmodified medium with α-methyl-NA, 1 μg/ml, 30 min, 37° C Initial restituted fluorescence intensity = +++

37° C								0° C			
15 min		30 min		60 min		180 min		60 min		180 min	
+++	(10)	+++	(3)	++(+)	(10)	+(+)	(5)			+++	(9)
(+)	(5)	+	(3)	(+)	(4)					+++	(3)
++	(3)			(+)	(3)					++(+)	(3)
+	(3)	(+)	(3)	0	(2)			+(+)	(5)	+	(5)
+	(2)			0	(2)					+++	(2)
(+)	(2)			0	(2)					++(+)	(2)

TABLE III. Fluorescence intensity of adrenergic terminals in irides of reserpine-pretreated rats after incubation with (+)-amphetamine and protriptyline

All incubations were made at 37° C. (Number of experiments within bracket.)

Drugs	Restitution of fluorescence by incubation for 30 min with α-methyl-NA				Disappearance of fluorescence after incubation in medium (without drugs) with only α-methyl-NA, 1 µg/ml for 5 ml or 0.1 µg/ml for 30 ml. Initial restituted fluorescence intensity = ++			
	1 µg/ml		0.1 µg/ml		15 min		60 min	
No drug	+++	(3)	++	(3)	++	(3)	+(+)	(3)
(+)-Amphetamine 10^{-5} M	++	(3)		(3)	+(+)	(3)	0	(3)
Protriptyline 10^{-5} M	+	(3)	0	(3)	++	(3)	(+)	(3)

In 3 expts. the fluorescence was restored by *in vivo* injection of α-methyl-NA after which the irides were incubated *in vitro* with (+)-amphetamine or protriptyline. The same observations were made as after *in vitro* restitution; this applied to both morphological appearance and effect of drugs.

Discussion

The appearance of an iris from an untreated rat was completely different to that where α-methyl-NA was taken up after reserpine pretreatment (Hamberger *et al.* 1964; Malmfors 1965). α-Methyl-NA was used in the present study instead of NA, since it was not necessary to pretreat the rats with a monoamine oxidase inhibitor. Normally the varicosities are extremely pronounced. This is probably because the main part of the endogenous NA is present in amine storage granules situated in the varicosities. After reserpine pretreatment, on the other hand, the terminals have a smooth appearance which suggests that at least part of the α-methyl-NA taken up exists outside the normal storage sites. The present experiments have shown that the same morphological appearance is observed after *in vitro* uptake as well. Incubation of an iris from a reserpine pretreated rat with α-methyl-NA, followed by further incubation at 37° C (but not at 0° C) without amine in the medium, changed the appearance of the terminals towards a more varicose picture. Similar morphological changes have been observed *in vivo* (Malmfors 1965).

These findings suggest that part of the α-methyl-NA taken up is bound to some intraneuronal structure with the same distribution as the amine storage granules. The amine storage granules may in fact be the site of this binding of α-methyl-NA, since isolated such granules seem to take up NA when it is present in high concentra-

Fig. 1 and 2. Irides of reserpine pretreated [illegible] incubated with α-methyl-NA [illegible] 37° C. Stretch preparations [illegible] either [illegible] Fig. 1 or after 180 [illegible] without axons in the medium. Fig. 2. Stretch [illegible] terminals without non-terminal axons (→) are visible both exhibiting strong fluorescence.

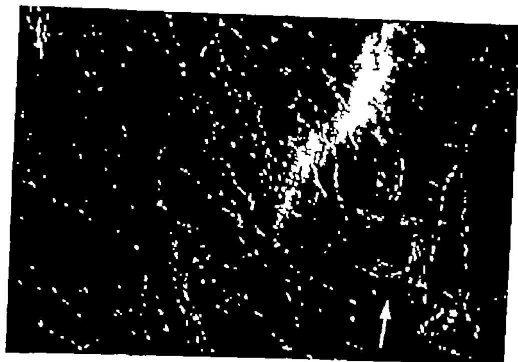


Fig. 3. Iris of reserpine-pretreated rat incubated with α-methyl-NA, 1 μg/ml 30 min, 37 °C and then further incubated in medium without am ne for 180 min, 37 °C. The nerv erminals hav prominent ricosities similar to those of untreated rat. Weakly fluorescent non-terminal xons (→) can also be seen. 400

Fig. 4. Iris of an untreated rat. Nerv terminals with intensely fluorescent ricosi ies are seen. A bundle of weakly fluorescent non-terminal axons (→) can also be recognized. 400 ×

tion in the medium, even after reserpine treatment (Stjärne 1964, Euler and Lishajko 1965). Moreover, it is highly probable that high catecholamine concentrations occur intraneuronally under the present experimental conditions. Reserpine resistant uptake of NA and α-methyl NA by the high-speed sediment of mouse hearts in vitro has also been observed (Lundborg and Stitzel 1967, Stitzel and Lundborg 1967).

Oxygen deprivation or addition of dinitrophenol (Green 1951), in combination with exclusion of glucose, prevented accumulation of α-methyl-NA (see also Hamberger 1967) and accelerated the disappearance of already accumulated amine. The effects were, in all probability, due to interference with energy-yielding processes, whereas the releasing effect of high concentrations of dinitrophenol at 0° C must be assumed to have a different mode of action.

Depolarization of the cell membrane by increasing potassium concentration prevented the uptake (cf. Hamberger 1967) and increased the disappearance of α-methyl-NA. It seems possible that this rapid release after depolarization is related to the physiological release of NA with nerve impulses. However, as nerve impulses seem to be ineffective in releasing NA which has been taken up after reserpine treatment (Malmfors 1965, Häggendal and Malmfors, to be publ.), it may be suggested that—apart from the depolarization itself—further, possibly energy-dependent mechanisms are involved in release of the transmitter (cf. Iversen 1967). This is substantiated by the finding that lowering of the incubation temperature ... release of α-methyl NA when the cell membrane is depolarized. The lack of ... generation at 0° C might also be related to the observation that the ... disappearance of α-methyl NA was prevented at this low temperature.

Both protriptyline and (+)-amphetamine inhibited ... the former being more potent (cf. Carlsson and Waldeck ... Hamberger 1967). When added to the incubation medium ... α-methyl NA, protriptyline moderately enhanced the ... complete inhibition of uptake of the spontaneously ... amine caused more rapid disappearance of the accumulated ... not only related to inhibition of uptake, since protriptyline ... probably to be ascribed to an active releasing action (cf. ... Malmfors, to be publ.). The release with (+)-amphetamine ... slower than with dinitrophenol or chemical depolarization ...

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Intracellular Ion Concentrations in Single Crayfish Axons

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Abstract

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A method for simultaneous determination of membrane potential and intracellular concentrations of sodium, potassium and chloride in single nerve axons is described in detail. Only 3—5 × 10 liter axoplasm is required for the ion analyses and consequently it has been possible to take 2 samples from the same axon. This allows for comparison of the intracellular ion compositions at different external conditions with good accuracy. The normal ion concentrations in crayfish giant axons are found to be (in mM ± S.E.) Na 17.4 ± 0.4, K 265 ± 1.8 and Cl 12.7 ± 0.4, indicating that probably none of the ions is distributed in electrochemical equilibrium. It is also shown that the distributions of sodium and potassium are directly dependent on an ouabain sensitive metabolic pathway but that this does not seem to be the case for chloride.

The determination of intracellular ion concentrations is of major importance for the study of nerve membrane function. Several investigations have been made on whole nerves (e.g. Fenn *et al.* 1934, Schmitt, Bear and Silber 1939, Krnjević 1955, Shaw and Simon 1955). Unfortunately these experiments have the drawback that a determination of extracellular space is necessary for evaluation of the results. It is difficult to find an ideal substance for extracellular space determinations and because of the uneven ion distributions a small error in extracellular space will give a considerable error in internal sodium and chloride concentrations. An additional complication is the Schwann cell layer which seems to have an internal composition different from that of the axon itself (Villegas, Villegas and Villegas 1965). If this is not taken into account in the determinations, more errors are introduced. The most reliable results have been obtained using single giant axons or extruded axoplasm from such axons, but even there the content of at least a whole axon has been used for a single determination of one or at most two ions (e.g. Bear and Schmitt 1939, Webb and Young 1940, Steinbach 1941, Steinbach and Spiegelman 1943, Keynes and Lewis 1951, Koechlin 1955, Keynes 1963, Brinley 1965).

This paper gives a detailed presentation of a technique that permits measurements of sodium, potassium and chloride in a single axon and correlates these con-

centrations to the simultaneously measured membrane potential. As it is also possible to take more than one sample from the same axon, a comparison can be made of the intracellular composition at different occasions. The results indicate that in crayfish axons none of the three ions appears to be distributed across the membrane in electrochemical equilibrium. Experiments with ouabain poisoning seem to indicate that the distribution of sodium and potassium, but not that of chloride is directly dependent on an ouabain-sensitive metabolic pathway.

Preliminary reports on this method have been presented at the XII Scandinavian Physiology Meeting, Turku, Finland and in Nature (Wallin 1966 a and 1966 b).

Methods

Material. The medial giant axon of the ventral nerve cord of the crayfish *P. ecambar clarki* has been used. The animals were transported by air from California, U.S.A. to Uppsala, Sweden and then kept in a water bath at temperatures varying between 6—10° C, depending upon the season of the year. No animal was used earlier than one week after arrival. The experiments were performed at room temperature (20—22° C) over one year with the exception of the period June—September.

Dissection. The ventral nerve cord was dissected out from the crayfish and mounted in a plastic chamber (Fig. 1) filled with physiological saline (van Harreveld 1936) of the following composition (in mM): NaCl 205, KCl 5.4, $CaCl_2$ 13.5, $MgCl_2$ 2.6, $NaHCO_3$ 2.3. pH was adjusted to 7.5 by adding small amounts of HCl. The connective tissue sheath surrounding the nerve cord was removed between ganglion 1 to 7 (counting distal from the head) and one medial giant axon was then isolated between ganglion 2 to 5. The ganglia themselves were only partially removed. The diameter of the medial giant axon varied between 125—175 μ but was sometimes even larger. There was no obvious relation between the diameter of the axon and the size of the animal. The length of the dissected part of the axon was 10—20 mm depending upon the length of the crayfish.

Electrode system. Membrane potential was continuously recorded through an annular micro-electrode bent L-shaped and filled with 3 M NH_4NO_3 + 10 mM NH_4Cl instead of 3 M KCl to avoid errors which might result from ion leakage from the micro-electrode. The tip of the electrode was about one micron or less, the impedance usually 10—30 megohms and the electrode was connected to an unit gain cathode follower through an Ag-AgCl electrode. Resting potential was recorded with a pen recorder and action potential monitored on an oscilloscope.

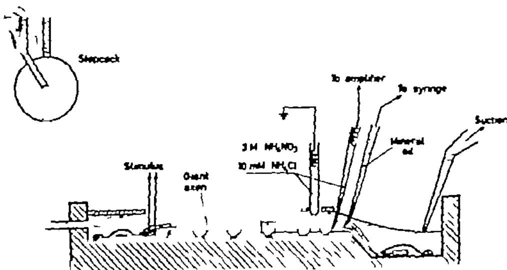


Fig. 1. Schematic picture of the experimental set up. The left part of the chamber is covered by a little plastic cover slip to prevent variations of the solution level.

Isolated giant axon
Mineral oil
Intracellular sample
Mineral oil
Potential recording electrode
3 M NH_4NO_3 10 mM NH_4Cl

Fig 2. The electrode system inside the giant axon. The injected oil drop lies in the left part of the axon. A sample of axoplasm has been taken and is enclosed by oil in the sampling pipette.

Resting membrane potential (mV)
Time
min
Injection of mineral oil
mV
mV
mV

Fig 3. Example of record of membrane potential obtained during an experiment. A the open arrow in the left part of the picture the electrode system is inserted and advanced to the recording position. When resting potential had stabilized he injection of oil was started. Before and after the injection and taking of sample action potential mplitude was measured on an oscilloscope and as indicated at the little black arrows the amplitude remained unchanged throughout.

I order to tak samples of the intracellular fluid, nother pipette was introduced into the axon together with he potential electrode This pipette as glass capillary pulled ou to tip of about 15—20 μ, bent L-shape and filled with white mineral oil (beosity 238 cs, refractn index 1 48 specific gravity 0.88) The tw glass pipettes were cemented ogether with insulating laquer (Insl-X E33 Insl-X Products Corpora ion, Yonkers, N.Y U.S.A.) and stored with the t ps in 3 M NH NO_3 + 10 mM NH Cl The sampling pipette w connected to an ordinary syringe through PVC-tube filled ith oil and with this rrangement oil could easily be injected into or sample taken from the axon

The reference lectrode was Ag-AgCl lectrode brough into ontact w th the bath la onically tapered agar bridge containing 3 M NH NO_3 + 10 mM NH_4Cl

Experimental proc d re A hole as cu in he isolated giant xon usually at ganglion al and the lectrode and sampling pipette were inse ted and ad anced to the recordi g position, which usually was between ganglion 3 and 4 See Fig 1 A 900—300 μ long drop of mineral oil as then slowly jected into the xon. The lectrode sys em hen retracted ht le and sample of 3 5 10 liter of intracellular fluid was sucked into he sampling pipett This was easily done since the cr f h axoplasm fluid and moved qui readily in the pipet The sample was hen enclosed by oil by moving the pipe p forw rd into the oil drop gain and sucking back some oil Fig 2 After thi the composition of he outside solution wa hanged or some other manipulation performed with the xon and after some time second sample w taken I some experiments when the f rst sample had been taken, silk lig ture was tied round the pipette close to the entrance hole hereby effects by leung off were, but in othe experiments this was omitted If all these manipula ions were done

fully no change of resting or ction potential occurred (Fig. 3). However if the injection of oil was done too rapidly it caused the resting potential to decline and occasionally the action potential was irreversibly abolished.

Analytical proc du e. Analyses of sodium, potassium and chloride concentrations were carried out as soon as possible after the samples had been taken.

a) *Sodium and potassium* were analyzed simultaneously in a integrating flame photometer (Öberg, Ulfendahl and Wallin 1967). The samples of axoplasm were blown out into a siliconized glass cup (Bilelad Clay Adams, New York, U.S.A.) and filled with mineral oil of the same type as used in the sampling pipett. From these mai samples   olume of roughly 0.7×10^{-9} liter was removed by   constant olume pipett and blown out i to little Teflon cup filled w th 0.65 cs silicone oil (Midland Silicone Ltd, Reading, England). For the construction of such pipettes see for example Ramsay (1950), Prager, Bowman and Vurek (1965). I the Teflon cup the diamete of the drop could be checked and t as then ttached to the platinum-iridium wire of the integrating flame photometer and analyzed. It was usually possible to make two or three analyses from the same main sample. For details of the proced re see Öberg t al. (1967). A calibration urve was made   advance of every experiment using a mixture of 70 mM NaCl and 400 mM KCl as standard sol tion. T test the reproducability of the instrument, 38 samples containing a mixture of pproximately 10^{-11} moles of potassium and 3×10^{-9} moles of sodium were analyzed giving a coefficient of ariation of ± 1.7 % for sodium and ± 3.3   for potassium. Other tests at different occasions ha   given coefficients of ariation between 1.5—4 %. Although this is an acceptable re producability systematic interference from other substances in the samples may nevertheles give fairly large errors. To avoid such errors, the compos ion of the tandard solution should be as close to that of the unknown solution as possible. However ery little is know about the composition of crayfish axoplasm and one would herefore ha   to investigate the effect on the nalyses of quite large number of substances. Some such tests were made and part from the effect of different anions (see Öberg t al. 1967 no important interference was found. I spit of this one cannot be absolutely sure of he absence f interference and therefore the following control method was utilized. When the od um and pot ssium concentrations of   sample had been determined still nother portion   taken from the same main sample and blown out int the Teflon cup. A know   olume of the standard solution was then added to this. The resulting total d op   as analyzed   nd from he result the percentage recovery f standard solution was   lculated. If he   on sample contained an nt rfering substance   a substance tha ga e errors in he analyses of sodium and potassium, this   terferent   or most of t) should hav   remained f er add tion of he   tra amount of salt. The percentage recovery aried from experimen to experiment but the mea   result of 32 recovery experiments was 105.8 % ± 1.0 (mean ± S.E.) for sodium and 100.1   ± 1.3 (me   ± S.E.) for potassium. In iew of this, all results ere corrected for by these f gures.

b) *Chlorid* was determined by lectrometric t ion using micro-method described b Ramsay Brown and Croghan (1955. For ach analysis bou 0.7 × 10 l ter as taken from the main sample nd ransferred d rectly to the microchloridometer. I w   usually possible to make tw   analyses from each main sample of xoplasm l every experiment.   alibr ion curve was made from know   concentrations of sodium or potassium chloride and in gene al the points proved t f t losely to   straight line   accordance with the findings of Ramsay t al. (1955). An example of such   calibr on curv   is shown in F g. 4. T   est the reproducability of the method, 50 samples containing equal amounts of hloride   pproximatel $1.8 \times 10^{-?}$ moles were nalyzed giving coefficient of   riation of ± 2.3 %. According to Ramsay et al. (1955) the result should be largely unaffected by other substances in l samples and this   as confirmed by the results of ecovery experimen   simila to hose d scribed for sodium and potassium. I   17 such experiments the recovery was 101.5 % ± 1 mean ± S.E.) and consequen ly all chloride alues er corrected accordingl

Stor ge f the samples. Some me hodological problems related to the handling of ery small olumes of fluid have been discussed by Öberg t al. 1967). An additional problem   how to store these small samples. Hellman Ulfendahl nd Walli   (1967) ha e show   ha oils ha   a tendency t take p   er and he fore he bo of oil   ry import   The white mineral oil used in these experiments reduced the olume of   phere containi g 4   l ter of water by 1 % in 30 min. Thi   not much bu   makes i difficul to tore phere samples in oil for any length of time. However if the samples are kept in the sampling pipette where the contact surface between oil and solution is small the w ter ptake is made negligible. Control experiment showed that   4 nl sample from a tandard sol tion did not ha ge its sodium and potassium concentrations during   4 hr   or ge   the sampling pipette. Th also demonstrated that no detectable xchange of sod um or pot   ium occurred   h the gas

The tip of which had been dipped in 100 mM NH   HPO

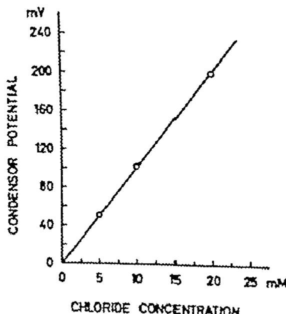


Fig 4 Calibration curv for chloride determination. Each point is the mean of tw determinations Pipette volume approximately 0.7 µl, i.e. the samples contained 3 5, 7 and 14 × 10 moles of chloride respectively

of the pipettes used. All samples er therefore stored in the sampling pipette until a few minutes befor the actual analysis The storage time was usually bet een 1— hrs and never exceeded 5 hrs.

Results

The collected results from 68 analyses of crayfish axoplasm with normal v Harre velds solution as external medium ($[K]_o$ = 5 4 mM) are shown in Table I If potassium and chloride wer distributed in electrochemical equilibrium across the membrane one would have expected an internal potassium concentration of 168 mM and internal chloride of 7 8 mM (assuming equal activity coefficients on both sides of the membrane) The difference between observed values and equilibrium concentrations is also indicated in Fig 5 where the distribution of the observed mem brane potentials is plotted against the simultaneously measured potassium (Fig 5 a) and chloride concentrations (Fig 5 b) No close correlation between the observed membrane potentials nd internal concentrations can be seen. This agrees w th the finding of Wall n (196 who show ed that the changes n internal concentrations were negligible when membrane potential was v ried more than 20 mV (from − 99 8 to   6 4 mV

T ABLE I Ion omposition of normal cr fish axoplasm Symbols V_m = membrane potential. [N] [K] [Cl]   ternal sodium, potassium nd chloride concentration

V (mV ± S E	[N] mM S E.	[K] (mM ± S.E.)	$[Cl]_i$ (mM ± S.E.)
86 6 ± 0 3	17 4 ± 0 4	265 ± 1.8	12.7 ± 0 4

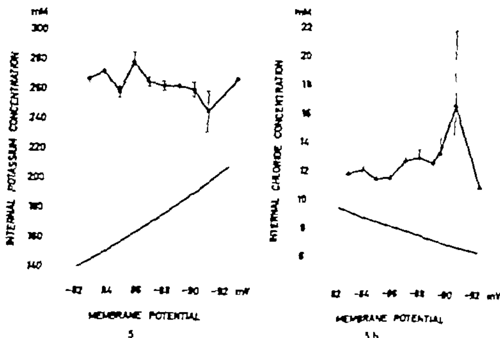


Fig. 5 Plot of the distribution of membrane potentials versus the simultaneously measured internal potassium (and chloride (b oncentrations External potassium concentration = 5.4 mM The upper curves are measured alues with ± S.E. for each point included as ertical bar The lower curves are the concentrations that ould have been expected if potassium chloride ere distributed in electrochemical equilibrium assuming equal activity oefficients on both sides of the membrane Data from 68 experiments

As the present technique could be used to take two samples from the same axon it was important to test whether or not the internal composition wa changed by taking the first sample or if progressive changes occurred with time In a series of experi- two samples were therefore taken from the same axon under identical external ditions. The time between the two samples wa aried between 3 and 121 mi ome experiment the entrance hole was left open but in others it was closed by a ligature. The esults are shown in Table II It is evident that the differences between the two samples are small. there is no clear increase of the changes with time and there seems to be no difference between the open or closed" axon If all experiments are grouped together he means differ by less than 6 % and according to the t-test the differences are not highly significant. Because of this, no correction for "background hange has been made in the results This does not exclude the possibility that a larger statistical sampling would reveal a mall but significant dif ference of a magnitude indicated in Table II between the two samples However even if a correction were made it would not in any wa change the interpretation of the results as presented here It has been noted earlier (Strickholm and Wallin 196 that the membrane potential of crayfish axons stays unchanged for rather long periods of time Brinley 1965 noted the same for internal concentrations in lobster axons. In contrast. it is often found that in squid axons, internal concen-

TABLE II. Control experiments where two samples of axoplasm have been taken under identical external conditions from the same axon with different time intervals. Symbols are the same as in table I. * Indicates that the pipette entrance hole was closed off by a ligature after the first sample had been taken

Time between samples (min)	V_m (mV)		$[Na]_i$ (mM)		$[K]_i$ (mM)		$[Cl]_i$ (mM)	
	Sample I	Sample II	Sample I	Sample II	Sample I	Sample II	Sample I	Sample II
3	−86.5	−85.5	18.5	18.3	270	253	14.0	14.9
4	−82.5	−83.0	21.4	24.0	266	288	11.0	11.5
15	−86.0	−86.0	23.8	24.7	282	263	11.5	10.3
26	−86.0	−84.0	18.1	19.4	260	262	10.5	11.1
30	−82.5	−83.0	15.0	15.7	264	262	8.2	8.1
49*	−86.0	−86.0	15.8	15.7	258	245	10.4	12.6
75	−88.5	−83.5	17.9	18.6	275	267	13.0	12.8
121	−87.0	−86.0	17.0	19.8	270	233	12.9	14.8
Mean	−85.6	−84.6	18.4	19.5	268	262	11.5	12.1
±S.E.	± 0.8	± 0.5	±1.0	±1.2	±3.0	±4.8	±0.7	±0.8
Percentage difference between sample II and I	+1.1 % P > 0.1		+6.0 % 0.05 > P > 0.02		−2.2 % P > 0.1		+5.2 P > 0.1	

TABLE III. The effect on membrane potential and internal concentrations of treating single axons with ouabain 10^{-5} M. Symbols as in Table I. Sample I is taken before and sample II after a certain time of ouabain treatment. In all experiments the axon was tied off close to the entrance hole after sample I had been taken

Time of ouabain treatment (min)	Diameter (μ)	V_m (mV)		$[Na]_i$ (mM)		$[K]_i$ (mM)		$[Cl]_i$ (mM)	
		Sample I	Sample II	Sample I	Sample II	Sample I	Sample II	Sample I	Sample II
22	150	−85.0	−84.0	14.8	17.7	283	270	10.2	10.1
30		−87.5	−78	13	18.2	255	246	9.3	11.4
36		−86.0	−84.0	13.0	20.7	251	250	11.4	13.8
43	198	−88.0		15.3	18.8	260	232	10.0	10.6
47		−81.0	−83.0	14.3	24.3	258	238	10.2	11.6
53	201	−88.5	−85.0	13.8	22.2	256	248	11.2	14.0
76	144	−89.0	−74.5	15.2	32.9	267	193	11.6	13.6
77	126	−87.0	−86.0	23.9	38.4	285	276	11.6	8.4
110		−85.5	−74.0	15.0	37.5	293	261	10.1	[illegible]

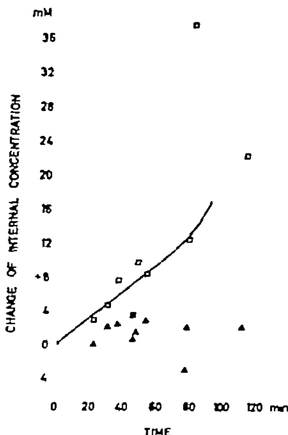


Fig 6 Effect of ouabain tre tment on the internal oncentrations of sodium (□ and chloride ▲. On the bscissa is plot ed time of ouabain treatment and on the ordinat change of internal concentrations (meaning increase of oncentration The hange of concentrations are calculated from T ble III The hea y solid line pproximates the change of internal sodium oncentrations nd is used for alculating sodi m influx See he ext

trations change progressivel with time St inbach and Spiegelman 1943 Keynes and Lewis 1951 A possible explanation may be that squid (but not cra fish and ter axons have small branches which are cut during the dissection allowing ternal sol tion to slowly leak in.

Effe t of uabai As the results of Tabl I seem to indicate that non of the three internal ions is distributed in electrochemical equilibrium across the mem brane a series of experiments were performed where the effect of 10 moles of ouabain was tested. It is well known that ouabain blocks the utilization of energy via the sodium and potas ium timulated ATP-ase system and also inhibit the action of the sodium extrusion mechani m in many cell for references see for example Glynn 1964 In analogy with earlier experiences one w uld therefore expect ouabain to cause an increase of internal sodium concentration and a de crease of potassium. If th chloride distrib tion also wa directly dependent upon metabolic energy one could possibly expect internal chlorid oncentration to de crease after the application of ouabain The result are presented in Table III Internal sodium concentration clearl increases progressively w th the duration of the ouabain treatment, and for potas ium the trend is opposite but with gr ater variability As in the case of potas ium on is mea ring rather small differences

between two large numbers, the variation is not surprising. Interestingly enough however the internal chloride concentration does not change much even when the sodium and potassium changes are large as in the 76 and 110 min experiments. Membrane potential does not change much either and if the electrochemical potential difference for chloride is calculated for all the experiments, the mean results for sample I and II will not be significantly different. The result seems to indicate then that when utilization of energy for the sodium extrusion mechanism is blocked an exchange of sodium and potassium occurs without any appreciable net chloride transport.

From these experiments one can also obtain an estimate of net sodium influx when the pump is blocked. In Fig. 6 the increase of internal sodium concentrations is approximated by the solid curve. Even though the later part of the curve is very uncertain, the initial slope is reasonably well defined and net sodium influx can be calculated. Assuming no volume changes occurring and utilizing 164 μ as the mean value of the measured diameters net sodium influx is 11.0 pmoles/cm² sec.

Discussion

The procedure outlined here has an advantage in that it provides information about membrane potential and internal ion composition in the same axon at two different occasions. That the results represent a physiological situation is supported by the observations which show no impairment of membrane function if one uses the resting and action potential as criteria. Secondly the experimental procedure does not seem to be more violent than internal perfusion of the axon which is now an accepted method for studying nerve function.

It is interesting to note that although in these experiments the axon lumen appeared macroscopically blocked by the oil drop, the conduction of action potentials across this obstructed segment was not interfered with. This indicates an electrical leakage path for the action current between the oil drop and the inner surface membrane suggesting that the injected oil is not in microscopic contact with the membrane and consequently does not affect the action potential. Other evidences indicating that axoplasm can be displaced or replaced without seriously affecting membrane function has been demonstrated earlier by the internal perfusion technique (Tasaki *et al.* 1962, Baker, Hodgkin and Shaw 1962).

In most electrophysiological investigations glass micro-electrodes filled with 3 M KCl are used for intracellular potential recording. In order to avoid any possibility of potassium chloride leaking from the electrode ammonium nitrate has been used instead in these experiments. As the mobilities of ammonium and nitrate ions are very similar the liquid junction potential between pipettes and internal and external solutions should be very small. Using Henderson's equation for liquid junction potentials the calculated difference in liquid junction potentials between using 3 M KCl or using 3 M NH_4NO_3 + 10 mM NH_4Cl filled electrodes is 1—2 mV. The values for membrane potential obtained with any of these two types of electrodes should therefore be quite comparable. This is supported by the

Dalton (1959) and Strickholm and Wallin (1967) who found resting potentials of —85 and —83 mV with 3 M KCl-electrodes in crayfish axons, which is comparable with the results obtained here.

The accuracy of the measurements of internal ion concentrations depends not only on the accuracy of the actual methods of analysis themselves, but also on the sampling procedure and the handling of the sample after it has been taken out These problems have been dealt with in the methods section and also by Oberg *et al* (1967) and Hellman *et al* (1967) It may seem strange that the composition of the axoplasm is affected so little by removal of a sample However the total length of axon available for sampling is about 2 cm (from metal clamp at one axon end to pipette entrance hole) whereas the length of the sample itself taking an average axon diameter of 150 μ is only about 230 μ Thus roughly one per cent of the available axoplasm is taken out and therefore the almost unaltered composition is not so surprising In spite of these considerations and although a change of internal composition is not statistically verified in Table II one should nevertheless be careful when discussing the significance of small concentration changes.

The values obtained here for internal ion concentrations with low sodium and chloride but high potassium concentrations are qualitatively similar to findings in other invertebrate nerves (see Brinley 1965) Quantitatively however both the value for internal sodium and chloride concentrations are much lower than found in earlier investigations on several different species. Thus from the table given by Brinley (1965) most values for internal sodium fall between 40- 100 mM and for chloride between 40—150 mM Part of the reasons for these differences may of course be species variation as well as differences in blood osmolarity In cases where whole nerves or whole isolated fibres have been analyzed, it is, however possible that the resulting sodium and chloride values have been too high, because of Schwann cells being analyzed together with the axon proper (*cf* Brinley 1965 Villegas *et al.* (1965) demonstrated that Schwann cell cytoplasm is rich in sodium and chloride compared to the axon itself and therefore this is a distinct possibility

The only earlier direct measurements from crayfish axons were made by Strickholm and Wallin (1965) who measured the internal chloride activity with a silver silverchloride electrode Assuming an internal activity coefficient of 0 .2 they found an internal chloride concentration of 35 mM ± 4 (mean ± S.E.) which is considerably higher than the value given here It is difficult to be sure of the reasons for this difference Strickholm and Wallin (1965) kept their crayfishes at room temperature whereas in the work presented here they were stored around 6 10 C and if the steady state temperature is of importance for ion distribution this may be a possible cause Secondly as is evidenced by the standard error the scatter in their series was more pronounced than that of this paper with some very high values. One cannot altogether neglect the possibility of defect electrodes as the cause of these values. For example if a microscopic crack were not detected in the glass insulation of the silver or platinum wire utilized the recorded activity would be in error

It has been mentioned above that the observed value of 12.7 mM for internal chloride concentration is higher than the 7.8 mM that would be expected if chloride were distributed in electrochemical equilibrium. As the absolute difference between the two values is rather small one could of course suspect a systematic experimental error. The reproducibility and sensitivity of the chloridometer is rather good (see Fig. 4) and therefore an error in the actual analysis is unlikely. It is also not very likely that a contamination or admixture of external solution has occurred, as one would then also expect a lower value for internal potassium. Another possibility is that the internal activity coefficient for chloride is about 60 % of the external, instead of being equal internally and externally as has been assumed. This cannot be excluded on the basis of the results presented here but some other observations make it rather unlikely. Additional studies (Wallin 1967) show that when external potassium concentration is increased, internal chloride concentration also increases, but in such a way that above $[K]_o$ = 10 mM chloride appears distributed in electrochemical equilibrium. Thus at $[K]_o$ = 16.7 mM internal chloride concentration was 18.5 mM with the corresponding equilibrium concentration being 18.7 mM. It is difficult to believe that an increase of internal chloride concentration of 6 mM should change the internal activity coefficient from 60 % to 100 % of the external.

In squid axons, Keynes (1963) has also found internal chloride concentration to be higher than the predicted equilibrium concentration. In addition he measured the activity of the extruded axoplasm and concluded that the internal activity coefficient was equal to that of the external solution. Furthermore Keynes could not demonstrate any effect of ouabain on chloride fluxes. This also agrees with the finding of this paper that in crayfish axons internal chloride concentration was largely unaffected by ouabain treatment. As far as can be judged from these facts, the distribution of chloride ions at normal resting potential is rather similar in squid and crayfish axons. In contrast to the behaviour of squid and crayfish axons with regard to ouabain, Casteels (1966) has recently demonstrated that in smooth muscle cells from the guinea-pig's taenia coli, internal chloride concentration decreases significantly when the muscle is treated with ouabain.

From Fig. 6 the net inward sodium flux after ouabain treatment was estimated to 11.0 pmoles/cm² sec. The results of Caldwell and Keynes (1959) indicate that about 1/6 of the total sodium efflux remains in squid axons when sodium extrusion is blocked by ouabain. If this is applicable to crayfish axons and assuming influx normally equals outflux, it would give a total unidirectional sodium flux of 13.2 pmoles/cm² sec in the resting crayfish axon. This is a fairly low value compared to sodium fluxes usually found in squid axons, 17—80 pmole/cm² sec (see Brinley and Mullins 1965) but is in reasonable agreement with the value of 5 pmoles/cm² sec given by Brinley (1965) for lobster axons.

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The Relation between External Potassium Concentration, Membrane Potential and Internal Ion Concentrations in Crayfish Axons

By

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Abstract

Wallin, B. G *The r lati n between external potassium co centration membrane potential and internal o oncentrations in rayfish axons* Acta physiol. scand. 1967 70 431—448.

In single crayfish xons simultaneous determinations were made of membrane potential and intracellular concentrations of sodium, potassium and chloride at different external potassium concentrations. When the external potassium level was varied from 2—150 mM, internal potassium concentration remained fairly constant. At the same time internal sodium concentration decreased with more than 50 % whereas internal chloride level increased 10 times. These ion changes ere accompanied by considerable volume increase. From the data it was calculated that net inward movement of potassium, chloride and w ter had occurred while slight outward transport of sodium was noted lower external potassium levels. When axon volume increased, observations were made indicating an increased intracellular hydrostatic pressure. I terms of the constant field equation, it appears as if the ratio P_{Cl} P_K and possibly also P_{X_A} P_K increases when the axon is potassium depolarized.

It is well known that the resting potential of squid axons is ery sensitive to variations in external potassium concentrations whereas it appears rather insensitive to sodium variations (Curtis and Cole 1942, Hodgkin and Katz 1949) From such experiments and also from radioactive flux measurements (g Keynes 1951 Brinley and Mullins 1965) and internal injection and perfusion experiments (Grundfest, Kao and Altamirano 1954 Baker Hodgkin and Shaw 1962 b) it is generally concluded that the resting squid a on is highly permeable to potassium ions but has ery low sodium and fairly low chloride permeability

Strickholm and Wallin (1967) studied the relation between resting membrane potential and external ion concentrations in crayfish giant axons with some unexpected results. As it is difficult to draw conclusions bout ion permeabilities from measurements of steady state membrane potentials they recorded the immediate potential change resulting from rapid external ion shifts. Although the

resting potential was fairly insensitive to chloride variations the sensitivity to chloride increased as the membrane was depolarized by potassium. In an attempt to quantitate the observations utilizing the constant field equation, some of the results, notably calculations of internal ion concentrations at elevated internal potassium concentrations, appeared in error. Because of this, and also because it is largely unknown how internal concentrations change in nerve when external potassium is varied, measurements of internal ion concentrations were undertaken. This paper describes the results of these measurements which allow the determination of membrane potential and internal concentrations of sodium, potassium and chloride in a single axon at different external concentrations of potassium.

The results show that when the crayfish axon is depolarized by high external potassium this leads to *inward movement of potassium and chloride* with a resulting *volume increase*. No inward movement of sodium occurs even when the resting potential is close to zero.

Preliminary reports on part of these results have been presented at the XII Scandinavian Physiology Meeting, Turku, Finland and in Nature (Wallin 1966 a and b).

Methods

Isolated medial giant axons of the crayfish *Procamba[illegible] [illegible]larkii* were used throughout. Dissection, insertion of electrode system, recording of membrane potential and the procedure of taking and analyzing intracellular samples has been described by Wallin (1967).

Sal[illegible] The normal physiological saline ([illegible] Harreveld 1936) had the following composition (in mM): NaCl 205, KCl 5.4, $CaCl_2$ 13.5, $MgCl_2$ 2.6, $NaHCO_3$ 2.3. In order to change the external potassium concentration sodium was exchanged for potassium mole for mole keeping total osmolarity constant. In each experiment one of the following changes was made from the normal external potassium concentration of 5.4 mM: [illegible] 20, 10, 16.7, 40, 75 or 150 mM.

Experimental proc[illegible] When the pipettes had been inserted and the oil drop injected into the axon the first sample was taken, al[illegible] under normal external conditions, and sealed off with oil. Then, in some experiments the axon was tied off with a silk ligature around the electrode shank near the electrode entrance hole but in most experiments [illegible] this omitted [illegible] point will be dealt with later. After this the potassium content of the external solution was changed and when the resting potential had attained a new constant value the second sample was taken. The time between samples was about 30 min in the lower ranges of external potassium concentrations but when external potassium was raised to 75 or 150 mM the potential did not become stable until after 45–70 min. In control experiments it was shown that if the time between samples was more than 30 min in the lower potassium ranges the internal concentrations did not change significantly. After the second sample was taken the external potassium concentration was brought back to normal and resting and action potential were measured again. If external potassium had been between 2–40 mM the recovery was almost always complete. However, when external potassium had been 75 mM only about 25 % of the axons recovered and from 150 mM no one recovered neither resting nor action potential. At $[K]_o$ = 75 mM no difference in internal concentrations was noted between axons that recovered and those that did not.

Volume measurements. To check calculated volume changes experiments were performed where axon volume was estimated on the basis of diameter measurements. From each ventral nerve cord both medial axons were dissected in the usual way, one of them was cannulated with a potential recording electrode and tied off close to the entrance hole by a silk ligature. If resting and action potentials were satisfactory in the cannulated axon the diameters of both axons were measured with an ocular micrometer (magnification 4[illegible]) at 8–10 different points along the length of the dissected segments. External potassium concentration was then increased from 5.4 to either 16.7, 40, 75 or 150 mM and when the membrane potential had stabilized on a new value new diameter measurements were taken.

TABLE I. The results of measurements of membrane potential and internal ion composition at different external potassium concentrations. (Symbols [Na], [K] and [Cl] is the concentration of sodium, potassium and chloride, respectively. Indices i and o refer to inside and outside solutions.

V_m = membrane potential, n = number of observations.)

1	2	3	4	5	6	
$[K]_o$ (mM)	V_m (mV ± S.E.)	$[Na]_i$ (mM ± S.E.)	$[K]_i$ (mM ± S.E.)	$[Cl]_i$ (mM ± S.E.)	$[Na]_i+[K]_i-[Cl]_i$ (= [X]) (mM ± S.E.)	
2.0	−99.8 ± 1.2	23.0 ± 3.2	249 ± 3.9	12.7 ± 0.8	259.3 ± 5.1	n = 6
5.4	−86.6 ± 0.3	17.4 ± 0.4	265 ± 1.8	12.7 ± 0.4	269.7 ± 1.8	n = 68
10	−76.4 ± 0.7	14.9 ± 1.0	256 ± 4.2	13.7 ± 0.6	257.2 ± 4.4	n = 5
16.7	−64.3 ± 0.8	16.6 ± 1.3	277 ± 6.4	18.3 ± 0.9	275.1 ± 6.6	n = 6
40	−42.8 ± 0.9	9.9 ± 0.6	256 ± 4.8	43.1 ± 0.7	222.8 ± 5.0	n = 8
75	−28.3 ± 0.3	12.0 ± 1.8	256 ± 3.8	73.8 ± 2.5	194.2 ± 4.9	n = 12
150	−13.1 ± 0.5	7.7 ± 1.4	246 ± 2.4	126.3 ± 2.3	127.4 ± 3.6	n = 6

Results

The collected results of potential measurements and analyses of internal concentrations in relation to the external potassium concentration are shown in Table I. All intracellular concentrations, except those at $[K]_o$ = 5.4 mM are obtained in the second sample from the axons. The values have not been corrected for any "background change" (compare Wallin 1967).

From the table it is clear that the intracellular *potassium* concentration remains fairly constant throughout the whole range of external potassium concentrations. As potassium is the main internal cation this might be expected if internal osmolarity is to remain constant. In Fig. 1 the measured concentrations of internal potassium are plotted against the simultaneously measured membrane potential and with the equilibrium concentrations included for comparison. Equilibrium concentration refers to the internal concentration that would be expected if potassium were distributed in electrochemical equilibrium (assuming equal activity coefficients on both sides of the membrane). It is evident that the measured concentrations are higher than the equilibrium concentrations except when the membrane potential is close to zero.

The internal *sodium* level, which already at normal external potassium is quite low decreases down to less than half the original concentration when the membrane potential changes from −86.6 to −13.1 mV. Hodgkin and Keynes (1955) have shown that sodium efflux increases when external potassium is elevated. The results of Table I clearly indicate that the sodium extrusion mechanism remains very effective even in a highly depolarized axon. One must, however, remember that with increasing potassium concentrations more and more of the sodium is substitu

resting potential was fairly insensitive to chloride variations the sensitivity to [illegible] increased as the membrane was depolarized by potassium. In an attempt to [illegible] the observations utilizing the constant field equation, some of the re[illegible] calculations of internal ion concentrations at elevated internal potassium [illegible] ration appeared in error. Because of this and also because it is largely [illegible] internal concentration change in nerve when external potassium is [illegible] of internal ion concentrations were undertaken. This paper describes the results of these measurements which allow the determination of membrane potential and internal concentration of sodium, potassium and chloride in a single axon at different external concentrations of potassium.

The results show that when the crayfish axon is depolarized by high external potassium this leads to [illegible] movement of potassium and chloride with a resulting [illegible] inward movement of sodium occurs even when the resting potential is close to zero.

Preliminary reports on part of these results have been presented at the XII Scandinavian Physiology Meeting, Turku, Finland and in Nature (Wallin 1966a and b).

Methods

Isolated medial giant axons of the crayfish *P. ... leniusculus* were used [illegible]. Dissection procedures of [illegible] system, recording of membrane potential and the procedure of [illegible] analyzing intracellular samples has been described by Wallin 1967.

Salines. The normal physiological saline (Van Harreveld 1936) had the following composition (mM): NaCl 205, KCl 5.4, CaCl 13.5, MgCl 2.6, NaHCO₃ 2.3. In order to [illegible] the external potassium concentration sodium was exchanged for potassium mole for mole keeping total osmolarity [illegible]. In each experiment one of the following changes was made from the normal external potassium concentration of 5.4 mM: to 20, 10, 107, 40, 75 or 120 mM.

Experimental procedure. When the pipettes had been inserted and the oil drop injected into the axon the first sample was taken. The [illegible] under normal external conditions and [illegible] led [illegible] Then in some experiments the axon was [illegible] with silk ligatures round the [illegible] but in most experiments this was omitted. [illegible] will be described later. After [illegible] the potassium concentration of the external solution was changed and the resting potential had [illegible] new constant value the second sample was taken. The [illegible] between [illegible] was [illegible] the lower [illegible] external [illegible] when external potassium was raised to 75 or 120 mM the [illegible] did not become stable [illegible] after 45–70 min [illegible] experiments showed that the time between samples [illegible] but 30 min [illegible] lower potassium [illegible] did not significantly. After the second sample [illegible] taken the external potassium concentration was brought back to normal and resting and action potential were [illegible] external potassium had been lowered to 40 mM the recovery was [illegible] samples. However when external potassium had been 75 mM only about 50% of the [illegible] and from 120 mM none recovered neither resting nor action potential. At [illegible] 75 mM no difference [illegible] concentrations was noted between axons that recovered and those that did not.

Volume measurements. To check [illegible] volume changes experiments were performed where axon volume was estimated on the basis of diameter measurements. From each central nerve cord both medial giant axons were dissected in the usual way, one of them was cannulated with a potential recording electrode and sealed off [illegible] hole by silk ligature. [illegible] resting and action potentials were satisfactory the unmutilated axon, the diameters of both axons were measured with a micrometer magnification 45 at 8–10 different points along the length of the dissected segments. External potassium concentration was then increased from 5.4 to either 10.7, 40, 75 or 120 mM and when the membrane potential had stabilized a new diameter measurement was taken.

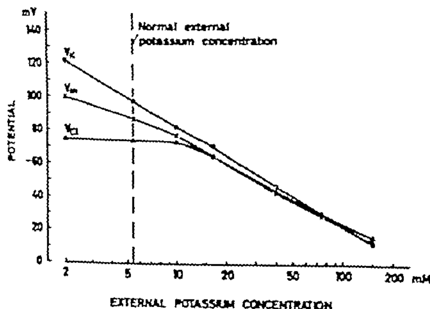


Fig. 2. Variation of observed membrane potentials (×) and equilibrium potential for potassium ( ) and chloride (Δ) with external potassium concentration. V_K is the equilibrium potential for potassium and is calculated from $V_K = RT/F \ln [K]_o/[K]_i$. V_{Cl} is the equilibrium potential for chloride and is calculated from an equivalent equation. In the calculations activity coefficients are assumed equal on both sides of the membrane.

internal activity coefficient remains unchanged at the different potassium levels, the general trend would of course be the same. However, until such measurements are made one must be very careful when discussing the implications of Table II.

In contrast to sodium and potassium the internal *chloride* concentration shows rather dramatic changes and it increases roughly 10 times while external potassium concentration varies between 2.0—150 mM. The main increase of chloride occurs above an external potassium concentration of 16 mM [illegible] when the membrane is depolarized more than 20 mV. This supports the observations by Strickholm and Wallin (196[illegible]) who found little change in internal chloride activity when external potassium concentration was shifted around the normal level. At high K_o-level however they noted larger internal chloride [illegible] shifts for a corresponding external potassium change. As discussed by Wallin (196[illegible]) the internal chloride concentration at normal resting potential [illegible] higher than would be expected if chloride were distributed in electrochemical equilibrium. However, when external potassium is raised above 10 mM the measured chloride concentration becomes almost identical to the calculated equilibrium concentration (Fig. 1). Although the absolute deviation from equilibrium concentration at normal resting level is only about 4—5 mM, with regard to potential the difference is more pronounced. This is illustrated in Fig. 2 where membrane potential and equilibrium potential for potassium [illegible] chloride are plotted against the logarithm of the external potassium [illegible]. In Fig. 2 it is assumed that activity coefficients for internal potassium [illegible]

are equal to those of the external solution (this assumption is discussed on page 12 and also by Wallin (1967)). It should be noted that the slope of the curves at higher potassium concentrations is close to 58 mV per tenfold increase in external potassium.

Keynes (1963) found that chloride probably was actively transported into the squid axon at normal resting potential. If this is true in crayfish axons too (no direct proof is available) the results presented here indicate that this transport is either inhibited or swamped by a passive permeability increase in the potassium depolarized axon.

Volume changes

The data for internal concentrations in Table I (column 6) indicate that at high $[K]_o$ the effective concentration of other internal anions than chloride must have decreased. Although other explanations could be put forward, the most likely is probably a change of volume of the axon. The size of such a volume change can be roughly estimated from the following electroneutrality condition which must hold at all times.

$$(1)\quad [Na]_i + [K]_i = [Cl]_i + [X]$$

here $[Na]_i$, $[K]_i$ and $[Cl]_i$ is the intracellular concentration of sodium, potassium and chloride respectively. It is assumed (a) that chloride is the only permeable anion and [X] then represents the equivalent concentration of all other internal anion (b) that no other internal ion concentration is changed to any significant degree.

As X is assumed impermeable the total *amount* of X in the axon is constant. By applying eq. (1) to the intracellular content at two different concentrations of external potassium, one will therefore from the resulting *concentration* of X obtain a figure for the volume change

$$(2)\quad \frac{Vol_2}{Vol_1} = \frac{[X]_1}{[X]_2}$$

where Vol is the volume and indices refer to situation 1 and 2.

Fig. 3 shows the results of such volume calculations for all external potassium levels and with the results from the direct microscopic volume measurements included for comparison. In the lower range of external potassium levels the curves agree very well. That the me[illegible]d volume change is smaller than the calculated at $[K]_o = 150$ mM is not ver[illegible]sing. One could in this situation clearly observe that the length of the axon had [illegible]sed, thus making the volume change estimated from diameter measurements onl[illegible] small. Secondly it is not inconceivable that this highly depolarized membrane [illegible]mes leaky, permitting some of the organic anions ([X] in eq. (1)) to escape from the axon. Such a loss would tend to make the calculated volume changes too big. The curves also agree with results obtained by Shanes (1946) and Lieberman and Wright (1966) on crab axons and Fenn

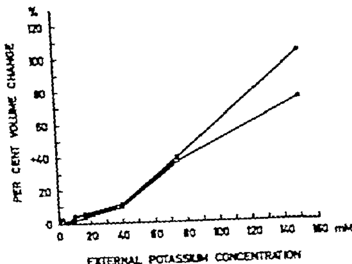


Fig. 3. Comparison between observed ( ) and calculated (●) volume changes with varying external potassium concentration. The calculated volume changes are obtained from eq. (2). The observed values are the means obtained from diameter measurements on four ([K]o = 162, 40 and 75 mM) or six axons ([K]o = 150 mM).

et al. (1934) and Freeman et al. (1966) have shown comparable results from lobster axons.

From the calculated volume changes one can obtain figures for net transport of ions utilizing

$$(3) \quad C_{tr} = Vol_2\,[C]_{i2} - Vol_1\,[C]_{i1}$$

where C_{tr} is the net transported amount of the ion in question [C ... is the internal concentration of the same ion with indices 1 and 2 referring to situation 1 and 2. If all transported ions are taken into account the sum of transported ... nions should equal the sum of transported cations.

Fig. 4 shows the results of such calc lations for sodium, potassi m ... nd chloride. The net transport for each ion in Fig. 4 is calculated from the d fference between the mean of the internal concentration ... the altered [K]o-value ... nd mean ... t the normal [K]o-value in the same axons ... Note ... he ... alues for intracell lar ... concentrations in Table I for the normal [K]o ... of 5.4 mM is the mean of all ... xons studied.) Therefore the exact numbers for Na_{tr}, K_{tr} ... nd Cl_{tr} ... annot be ... d ... ed from Table I. Furthermore as in most ... xons ... lume meas rement ... were not made the numbers for Na_{tr}, K_{tr} and Cl_{tr} are gi en as m llimoles per liter axoplasm ... f the final ... olume ... Vol ... Fig. 4 demonstrates that when the membra ... e ... potass ... r depolarized a net inward transport of potassium ... nd ... hloride ... ... addition possibly a small outward transport of sod um ... t least ... t lower ... ... f external potassium. The ... alculated ... lume curv ... is also incl ... ded in the f ... ... nd it approximately follows ... he potassium chloride thereby ma ... ... osmolarity intracellularly.

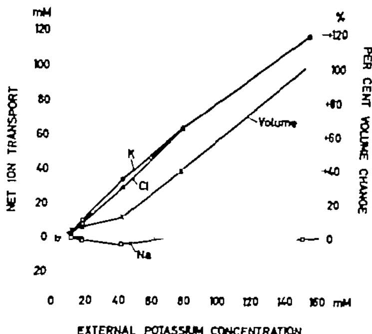


Fig. 4 Calculated net ion transport (eq. 3) and volume changes (eq. 2) versus the external potassium concentration. Calculations are based on observed values for internal ion concentrations. Positive sign means transport into the axon. The net ion transport is expressed in millimoles per liter axoplasm of the final volume (Vol$_f$). For further explanations see the text.

In early experiments utilizing this technique it was thought that the widening shank of the glass pipettes sealed off the hole in the axon and that a volume change would result in a diameter change. This may be true if the volume changes are small and if the pipettes are not moved, once they have been positioned. However for the sampling procedure it was necessary to move the pipette a little back and forth and when the volume changes were big as at $[K]_o$ = 75 or 150 mM it became very clear that axoplasm was leaking out through the entrance hole. The evidences were that the diameter of the axon did not increase very much and that the oil drop started moving out towards the opening after the shift of external solutions. The situation is illustrated in Fig. 5 situation 1. When the membrane potential had stabilized on a new value the oil drop also stopped moving and remained in a position somewhere between the pipette tip and the opening of the axon. Fig. 5 situation 2. As the pipette tips were in the enclosed region to the left of the oil drop, no admixture of external solution had to be feared but a dilution of the axoplasm had occurred. The problem arises here whether the volume and ion transport figures from an axon that has a leak in one end represent a true steady state. One may argue that if there exists a difference in colloid osmotic pressure between inside and outside solutions no hydrostatic pressure can develop inside an open axon to balance such a difference because axoplasm would flow out of the

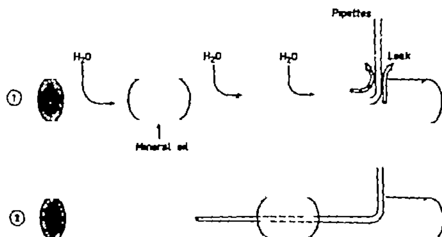


Fig. 3. Schematic picture of the olume flow in an "open axon when external potassium concentration is increased. Situation 1 illustrates the transient phase when the oil drop is moving towards the pipette entrance hole and situation 2 is the new steady tate when the oil drop has stopped moving.

open axon till the difference had disappeared. In order to check whether or not there would be any differences between the intracellular concentrations in an open or closed" axon, experiments were done both at $[K]_o$ = 75 and 150 mM with the axon closed off near the entrance hole by a silk ligature. The results at $[K]_o$ = 75 mM are summarized in Table III. No important difference between the closed nd open axon was found and consequently the mean value of all observa tions have been used in Table I The result of these experiments suggests that although one cannot exclude the bsence of real steady state in the open axons, the deviation is fairl small and is not detected with the measurements of internal concentration made here

When the axon was tied off the axon diameter increased ery clearly when ex ternal potassium was ele ted and the movement of the oil drop disappeared or t moved very slighth in th opposite direction In some experiments, when the second sample was taken and the sample was to be enclosed by oil it was necessary to cut the ligature to be able to mov the pipett into the oil drop. On these occasions it was noted that as soon as th ligature was cut th oil drop started moving rather rapidly out towards the opening in the axon In one experiment where $[K]_o$ had been raised to 150 mM, the oil drop even flowed out through the opening This seems to be lear evidence for a positiv h drostatic pressure ha ing developed inside the axon That this should oc ur seems ra her reasonabl in iew of the fact that the axon w considerably swollen under these circumstances and w th the axon being enclosed by la er of Schwann ells and connecti tissue one wou d not expect this struct re to ha perfect plastic properties. It ha been shown earlier by internal perfusion experiments (Baker et al 1962 a that in squid axons a hydrostatic pressure difference of 20–30 mm H_2O between nside and outside sol

TABLE III. Comparison between intracellular ion concentrations in open and closed" axons. $[K]_o = 75$ mM

	V_m (mV) open	closed	$[Na]_i$ (mM) open	closed	$[K]_i$ (mM) open	closed	$[Cl]_i$ (mM) open	closed
	—29.5	—28.0	15.2	9.6	278	244	65.0	70.0
	—	—28.5	7.8	29.4	274	240	66.0	97.5
	—27 0	—28.5	16.1	7.5	258	244	75.9	69.5
	—28.0	—29.5	9 4	12.8	278	254	69.0	82.5
	—27 0	—27.5	10.5	8.0	255	251	70.2	71 7
		—28.5		8.7		253		75.4
		—29 0		8.8		248		73.4
Mean	—27.9	—28.5	11.8	12 1	268	248	69.2	77 1
±S.E.	± 0 6	± 0.3	±1.6	±3.0	±5.3	±2.0	±1.9	±3.8
	P>0 1		P>0.5		P<0.01		P>0.1	

was needed to keep the axon inflated to its normal size If the hydrostatic pressure was higher than 50 mm H_2O the action potential started to fail The observations mentioned here seem to indicate that variations of hydrostatic pressur can occur in nerve axons. As hydrostatic pressure variations play an important part in Teorell's oscillating membrane model (see for example Teorell 1962) it has been attempted to make quantitative measurements of pressure variations in these cells Preliminary results (Lieberman and Wallin) seem to confirm that an increase of external potassium causes an increase of hydrostatic pressure in crayfish axons.

Discussion

Volume relations

In Fig 6 a simplified picture is given of the ion relationships in these axons t $[K]_o = 5.4$ mM (a) and $[K]_o = 150$ mM (b) A simple model for describing the relation between ion concentrations and volume is the Donnan equilibrium, used for example by Boyle and Conway (1941) Assuming potassium and chloride as the only permeable ions and assuming equal total concentrations and hydrostatic pressures on both sides of the membrane and also neglecting internal sodium, the Donnan equilibrium implies that

$$(4) \quad \frac{[K]_o}{[K]_i} = \frac{[Cl]_i}{[Cl]_o}$$

and from eq (2) and (4)

$$(5) \quad \frac{Vol_2}{Vol_1} = \frac{([Na] + [B])_1}{([Na]_o + [B])_2}$$

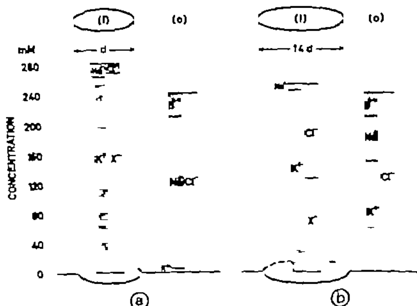


Fig. 6 Simplified picture of the relation between internal and external ion concentrations in crayfish axons at $[K]_o = 5.4$ mM (a) and $[K]_o = 150$ mM (b). B^{++} represents the sum of calcium and magnesium ion equivalents. External bicarbonate ions included in the chloride column. d is the diameter of the axon at $[K]_o = 5.4$ mM. The diameter at $[K]_o = 150$ mM obtained from the volume given in Table IV, column (3).

[B] in eq. (5) represents the sum of external magnesium and calcium ion equivalents.

It is rather obvious (cf. Fig. 2) that for the situation in Fig. 6 (a) relation (4) does not hold, but that it is approximately correct for high concentrations of external potassium as in Fig. 6 (b). However, Table IV shows that the volume ratios can be estimated fairly accurately from eq. (5) as well as from eq. (2) which was based on impermeability of the X ions and did not require a Donnan assumption. This seems to suggest that quantitative measurements of volume changes only would not distinguish a Donnan situation from the existing ion distribution pattern.

b) *Potential and permeability relations*

In order to describe the variation of steady state membrane potential with external ion concentrations, Hodgkin and Katz (1949) showed that a good agreement could be obtained using the constant field equation, originally derived by Goldman (1943)

$$V_m = -\frac{RT}{F}\ln\frac{P_{Na}[Na]_i + P_K[K]_i + P_{Cl}[Cl]_o}{P_{Na}[Na]_o + P_K[K]_o + P_{Cl}[Cl]_i} \qquad (6)$$

where V_m is the membrane potential, P_{Na}, P_K and P_{Cl} is the permeability of sodium, potassium and chloride respectively and [Na], [K] and [Cl] are the corresponding concentrations with subscripts i and o referring to the inside and outside.

TABLE IV. Comparison of volume ratios calculated from eq. (5) and (2) and observed from diameter measurements at different external potassium levels. Vol_0 is always the initial volume at $[K]_o = 5.4$. Vol_i is the volume at any of the elevated $[K]_o$-levels

(1) $[K]_o$ (mM)	(2) Vol_i/Vol_0 (eq. 5)	(3) Vol_i/Vol_0 (eq. 2)	(4) Vol_i/Vol_0 observed
5.4	1	1	1
10	1.02	1.04	—
16.7	1.05	1.06	1.03
40	1.16	1.11	1.10
73	1.41	1.39	1.37
150	2.52	2.02	1.73

A correct utilization of eq. (6) requires ion activities being used rather than concentrations. The obvious weakness of the following treatment is the lack of knowledge of internal activity coefficients in these axons. From the result of Hinke (1961) and Lev (1964) it may be suspected that the internal activity coefficient for sodium is lower internally than externally. As internal sodium concentration is very low this will, however, not greatly affect the results. Hinke and Lev did not find any large difference in activity coefficients for potassium between inside and outside solutions and Keynes (1963) got similar results for chloride. Their results form the justification for carrying the discussion through assuming activity coefficients equal on both sides of the membrane. Nevertheless, until actual measurements of internal activities have been made in this nerve the conclusions must be regarded as tentative.

If chloride is distributed in electrochemical equilibrium eq. (6) reduces to

$$(7) \quad V_m = -\frac{RT}{F}\ln\frac{P_{Na}[Na]_i + P_K[K]_i}{P_{Na}[Na]_o + P_K[K]_o}$$

As the results of the intracellular analyses (Table I and Fig. 2) indicate that in crayfish axons chloride is approximately in electrochemical equilibrium from $[K]_o = 10$ and up eq. (7) is applicable for these situations. Inserting the measured values of membrane potential and internal concentrations, the permeability ratio P_{Na}/P_K can be obtained for the various experimental points, and they are summarized in Table V.

The calculation of P_{Na}/P_K for Table V contains the expression $[K]_i - [K]_o$ exp $(-V_m F/RT)$ and when $[K]_o$ increases this expression will be close to zero (indicating that potassium is close to an equilibrium situation). Consequently at the high $[K]_o$-levels small experimental errors in $[K]_i$ and V_m will give large errors in P_{Na}/P_K calculated in this way and therefore one should be careful when evaluating table V. With this in mind, the six times increase in P_{Na}/P_K ratio between $[K]_o = 10$ and $[K]_o = 75$ mM becomes rather uncertain. An increasing trend is, however, possible and as illustrated in Fig. 7 it could such escape attention in a

TABLE V The ratio P_{Na} P_K at different external potassium levels calculated from eq (7)

$[K]_o$ (mM)	V_m (mV)	P_{Na} P_K
5.4	—86 6	(<0.015)
10	—76.4	0.011
16.7	—64.5	0.024
40	—42.8	0.040
75	—28.5	0 062
150	—13.1	<0

The value for P_{Na} P_K at $[K]_o$=5.4 mM is maximum value obtained from eq (6) If P_{Cl} P_K is to be positive number P_{Na} P_K must be less than 0 015.

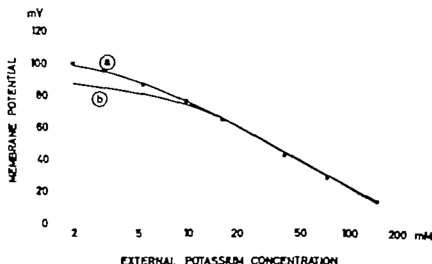


FIG. 7 Plo of observed membrane potentials at different external potassium concentrations ( ) Curve ( ) is calculated from eq (6) utilizing he observed alues for internal ion con centrations and the permeability ratios P P P = 0 011 1 0 1 Curv (b) is calculated in the same way as ( ) but with permeability ratios being P P_K P ₁ = 0 011 1 1

graph of membrane potential ersus the logarithm of external potassium concentration. In Fig 7 curv (a) is obtained from eq (6) using the experimental values for internal concentrations but w th constant permeability ratios (P P_K P = 0 011 1 0 1) This confirms that the P_N P_K ratio on.y ery slightly affects th potential alues at higher external potassium levels (cf Adrian 1956

It is also llustratn to consider the influence of the chloride permeabil ty Abov $[K]_o$ = 10 chloride is in electrochemical equilibrium nd eq (6) impl fies into (7) A onsequence of this is that the size of P_{Cl} does not ffect the potential curv abov $[K]_o$ = 10 mM This is shown by curv (b) in figure 7 which is

axons. The two groups may have a somewhat similar relative chloride permeability at normal resting potential but part of the *conflicting evidences could be explained* if in squid axons the relative chloride permeability does not increase with depolarization as was suggested for crayfish axons by Strickholm and Wallin (1967). Very little can be said about the functional significance if any of such a *difference*. Squid is a mollusc, whereas crayfish, lobster and crab are arthropods and therefore it may be of phylogenetic origin. Structurally squid and lobster axons have a rather similar appearance although they show some differences in the Schwann cell and connective tissue layer (Freeman *et al.* 1966). To judge from voltage clamp and flux measurements the excitation process seems to be similar in the two groups (Julian *et al.* 1962 b. Brinley 1965).

Boyle and Conway (1941) studied the effect of increasing the external potassium concentration on frog striated muscles and they found an unchanged internal potassium concentration but an increased cell volume and also an increased concentration of internal chloride. Adrian (1956) confirmed their findings and later on Hodgkin and Horowicz (1959) demonstrated more directly that striated muscle cells have a high relative chloride permeability. The results obtained here are *qualitatively similar to the results from striated muscle cells in that crayfish axon* take up potassium, chloride and water when depolarized by increasing external potassium. On the other hand, Casteels and Kuriyama (1966) has demonstrated that the situation is different in smooth muscle cells of the guinea pig's taenia coli. There an increase of external potassium up to 20 times did not change internal chloride concentration and cell volume. It is, however, interesting to note that there is no difference in the behaviour towards sodium ions. Both in striated muscle cells (Adrian 1956), smooth muscle cells (Casteels and Kuriyama 1966), heart muscle cells (Haas, Glitsch and Kern 1966) and the crayfish axons used here the internal sodium concentration decreases when the membrane is depolarized by high external potassium. It seems to demonstrate that a high membrane potential is no requirement for the function of the sodium extrusion mechanism. That a membrane potential is not required would be expected if the sodium and potassium activated ATP-ase from crab axon homogenate described by Skou (195[illegible]) *is to be identified* with the sodium pump as advocated by Baker (1965).

At normal resting potential ($[K]_o$ = 5.4 mM) the ratio $P_X : P_K$ in these axons *seems to be less than* 0.015 (see table V). Compared to the values obtained for squid axons, for example 0.04 given by Hodgkin and Katz (1949), this is rather low. On the other hand it agrees well with the results of Brinley (1965) for lobster axons. More interesting however is the possibility of permeability ratios increasing with decreasing membrane potential as suggested in tables V and VI. This has been noted before e.g. by Baker, Hodgkin and Meves (1964) who found an increasing $P_X : P_K$ ratio when the squid axon was depolarized by internal perfusion with decreasing potassium concentrations. According to the recent work of Sandblom and Eisenman (1967) this is to be expected when the ionic strength is not kept constant and equal on both sides of the membrane as in those experiments. Sand-

blom and Eisenman also show that if the membrane is permeable both to anions and cations the permeability ratios of the resting membrane can vary with membrane potential even though ionic strength is constant. The results of this paper and of Strickholm and Wallin (1967) seem to be in agreement with these theoretical considerations.

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Uptake of Monoamines by Mouse Peritoneal Mast Cells in Vitro

By

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Using radioactive isotopes, Furano and Green (1964) showed that mast cells take up exogenous histamine (H) 5-hydroxytryptamine (5-HT) *in vivo* and *in vitro* but not dopamine (DA) or noradrenaline (N) Adams-Ray *et al.* (1966) presented evidence that mast cells of the hamster ear take up *in vivo* dihydroxyphenylanine (D) and 5-hydroxytryptophan (5-HTP) but not DA, while Eränkö and Kauko (1965) demonstrated that mesenteric mast cells of the mouse take up not only 5-HT and D but also DA and N *in vivo* Day and Stockbridge (1964) found that ascites tumour mast cells take up 5-HT H, N and tryptamine from the medium. The present paper deals with the amine uptake by normal mouse peritoneal mast *in vitro*

The cells were obtained by injecting 2—3 ml of the Krebs salt solution, pH 7.0 supplemented with calcium chloride and glucose (Eränkö and Räisänen 1966) into the peritoneal cavity The fluid was then collected and the cell suspension incubated at 37° C in the salt solution, into which the amines were added dissolved in the salt solution. The total incubation volume was 10 ml with about 4—7 × 10⁴ cells/ml, both mast cells and leukocytes. After centrifugation at about 10 g for 10 min, the cells were washed 4 times by resuspension in 3 ml of Krebs salt solution without added amine and collected by centrifugati n. Smear preparations of the sediment were then exposed to paraformaldehyde vapour to make the amine-concentrating cells fluorescent (see Eränkö and Kauko 1965) After fluorescence photomicrography the preparations were stained with toluidine blue and rephotographed in transmitted light

The results are presented in a schematic form in T ble I. It is obvious from it that 5-HT was taken up much more readily than any of the other substances tested, suggesting a specific uptak mechanism for this amine in the peritoneal mast cells. Fluorescence observed after incubation with 5-HTP may have been due in part to conversion into 5-HT Leukocytes ther than mast cells, i.e. cells not stainable with toluidine blue did not becom fluorescent after incubation w th any of the substances tested.

The observations described indicat that the peritoneal mast cells of the mouse contain very little, if any intrinsic D DA, N 5-HTP or 5-HT However all of

TABLE 1 Intensity of formaldehyde-induced fluorescence in peritoneal mast cells after incubation in different concentrations of amine and amine precursors

Substance added	Concentration (μg/ml)				
	0.01	0.1	1	10	50
None (control)	—	—	—	—	—
Dihydroxyphenylalanine	—	+	++	+++	+++
Dopamine	—	++	+++	+++	+++
Noradrenaline	—	+	++	++	+++
5-hydroxytryptophan	—	—	+—	+	++
5-hydroxytryptamine	+	+++	+++	+++	+++

Explanations — no fluorescence, + weak, ++ moderate, +++ intense fluorescence

these compounds are taken up by them from the incubation fluid *in vitro*, especially 5-HT whose uptake was marked even with low amine concentrations. Studies are in progress to further investigate the specificity of this uptake mechanism.

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Tachyphylaxis in Rat Uterus to Some Prostaglandins

By
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Tachyphylatic response to prostaglandins has been observed on isolated smooth muscle organs like rabbit and rat uterus (Eliasson 1959) cat duodenum and guinea-pig ileum (Änggård and Bergström 1963) but also on brain stem neurones (Avanzino, Bradley and Wolstencroft 1966) and isolated perfused rat heart (Vergroesen, Boer and Gottenbos 1967) Eliasson (1959) noted that the isolated rabbit and rat uterus could show a tachyphylactic response to prostaglandins E_1 and F α without having the same response pattern to the partially purified prostaglandin extract. In the present study the reactivity pattern of isolated rat uterus to prostaglandins E_1, E_2 and F α is reported.

The experimental conditions were as described by Eliasson (1959) The reactivity of the uterine horns was first tested with repeated doses of oxytocin and the load was adjusted until a good dose-response relationship was obtained. The stimulating drug was left in contact with the organ until the maximum height of contraction had been passed. Washing was done twice and there were three minutes between the applications of the drugs.

In relation to PGE_1 (= 1) the activity of PGF α was about 1.2 (range 0 9—1.55) Due to the occurrence of tachyphylaxis, the ratio is only approximate.

PGE was tested in 11 PGE_2 in 6 and PGF α in 8 expts. In all there was a marked tachyphylactic response to PGE_1 (Fig 1) The uterus showed tachyphylaxis against PGF α in six of eight and against PGE_2 in two of six expts. After a marked tachyphylaxis had been recorded for PGE_1 the response to a following dose of PGF α appeared normal and vice versa (Fig 1) Thus, there was no cross-desensitization between these two prostaglandins.

When the sensitivity to a given prostaglandin had been depressed, it could also be restored to normal after in or in some cases repeated application of another smooth muscle stimulating substance e g oxytocin, vasopressin.

In 4 expts. we noted a marked tachyphylaxis to PGE_1 without any concomitant decrease in the response to PGE_2 (Fig 2) In one experiment it appeared possible that tachyphylaxis to PGE_1 was accompanied by a suppression of the response also to a subsequent dose of PGE_2.

The observation that the isolated rat uterus, after having developed a decreased sensitivity t PGE_1 or PGF α still responds normally to the other prostaglandin

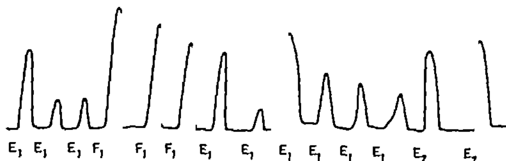


Fig. 1 Tachyphylactic response in isolated rat uterus to prostaglandins E and $F_{2\alpha}$. Bath volume 5 ml. Temperature 30 C. E_1 = 0.8 μg PGE_1/ml and $F_{2\alpha}$ = 1.2 μg $PGF_{2\alpha}$/ml. Note that tachyphylaxis for one prostaglandin does not decrease the response for the other compound.

Fig. 2. Isolated rat uterus showing marked tachyphylactic response to PGE_1 (0.6 μg/ml) without having the same reactivity pattern for PGE_2 (1.2 μg/ml)

strongly indicates that the rat myometrium contains different receptors for these two compounds. Whether there are specific receptors also for PGE_2 is not clear but some of our results support such a view. Different actions for PGE_1 and PGE_2 have also been reported by Kloeze (1967) who found PGE_1 to inhibit the adenosine diphosphate induced aggregation of platelets from man, pig and rat while PGE_2 in the same dose range enhanced the aggregation of the platelets from pig and rat. It is thus obvious that a two-receptor (i.e. E- and F-receptor) theory for prostaglandin actions does not fit all experimental findings available (*cf.* Pickles 1967).

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Quantitative Studies on the Transmitter Release at Adrenergic Nerve Endings

By

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Results obtained during studies of mechanisms involved in the elimination of the adrenergic transmitter (Folkow, Häggendal and Lisander 1967) together with figures for the noradrenaline (NA) content in adrenergic nerve varicosities (Dahlström, Häggendal and Hökfelt 1966) seem to allow an approximate deduction of the NA amount, released per stimulus at the individual neuroeffector junction.

In anesthetized cats all the vasoconstrictor fibres to the calf muscles were electrically stimulated at 6 imp/sec during a state of nearly maximal exercise hyperemia under steady control of the calf blood flow. The NA amounts in arterial blood and in the venous effluent were measured fluorimetrically (for details see Carlsson, Folkow and Häggendal 1964).

The adrenergic transmitter is considered to be inactivated by principally three mechanisms: 1. Reuptake into the nerve varicosities by a membrane "pump" mechanism, 2. Local enzymatic destruction, probably mainly by catechol-O-methyl transferase (COMT), 3. Escape to the blood stream. — The first mentioned mechanism appears under certain conditions to be of dominant importance. However, during exercise hyperemia the NA escape to the blood stream generally increased greatly and administration of COMT and monoamine oxidase inhibitors did not further increase this NA "overflow" significantly. On the other hand, addition of "membrane pump" blockers in small or moderate amounts was found to further enhance the NA "overflow", while large concentrations of such drugs appeared to suppress the NA release.

The highest NA amounts found to be released were usually around 100 ng per 100 g calf muscle for a period of 100 sec of stimulation at a rate of 6 imp/sec; occasionally values some 50 per cent higher were obtained. It is likely that these amounts reflect nearly the total amount of transmitter released, since NA would in this situation be eliminated practically only via the blood stream. If so, 0.15—0.25 ng NA would be released per stimulus from 100 g of muscle which, in total, contains about 8000 ng. It follows that, in round figures, 2—4 · 10⁻ of the total NA content is released per stimulus. This figure may be compared with that for the cat's adrenal medulla, which can be calculated from Celander's (1954) data to correspond to a release per stimulus of about 6—8 · 10^{-4} of the total catechol amine content in this

Practically all NA in skeletal muscle is present in the varicosities of the vaso constrictor fibres (Fuxe and Sedvall 1965) and on activation of an adrenergic neuron NA appears to be released from all its varicosities (*cf* Malmfors 1965) The present technique for stimulation will activate virtually all the vasoconstrictor neurons of the calf muscles, hence essentially all the adrenergic varicosities. Thus, the fraction 2—4 10^{-} may as a rough average hold also for the individual varicosity

The NA content per varicosity is about 5 10^{-3} pg in the rat (Dahlström, Häggendal and Hökfelt 1966) and suggested to be the same in the cat (Dahlström and Häggendal 1966) One impulse would then release about 1—3 10^{-} pg Since the weight of the NA molecule is 2.8 10^{-22} g, one impulse would release around 500—1000 NA molecules from each varicosity

Clearly these figures must be considered as very approximate, but they nevertheless make it likely that only a *fraction* of the NA content in a varicosity granule is released per stimulus, since each varicosity contains on an average only some 1500 granules, at most (Dahlström, Häggendal and Hökfelt 1966) The calculated amount released per stimulus, would correspond to less than 5 per cent of the NA content in one granule.

Even if only 500—1000 NA molecules are released per stimulus from the average varicosity this might nevertheless imply a very high concentration at the neuroeffector junction, at least at sites where the junction gap is narrow Suppose the "contact" area is 1—2 μ and the gap say 1000 A, concentrations of the order of 1 μg/ml would occur being several hundred times higher than those ever reached in the arterial blood at intense physiological activations of the sympathetic system However it is not known how large a fraction of the α-receptors that are directly exposed to such transmitter concentrations, neither whether these concentrations evoke graded or all-or none responses of the directly innervated vascular effectors. Direct parallels to the nervous control of skeletal muscle or distinct "motor units" ooth muscle cannot *a priori* be drawn.

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